Aspects of biliary atresia and other pediatric cholestatic diseases

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Paranimfen

Astrid Klooster

Yunwei Wei 魏云巍

To my family

Contents

CHAPTER 1

Introduction and outline of the thesis

Introduction and outline of the thesis

Biliary atresia (BA) is an obliterative disorder of unknown etiology that affects both the intra- and extra-hepatic bile ducts. The incidence is approximately one in 10,000 live births world wide (1). Anatomically, it can be classified into three types according to the location of the obliteration of the biliary tree (2). Type 1: obliteration of the common bile duct; Type 2: atresia of the hepatic duct; Type 3: atresia of both the right and left hepatic ducts with obstruction of the porta hepatis. More than 90% patients belong to type 3. Another classification is based on the presence or absence of other malformations and might be associated with the period in which BA develops. Following this classification there are two types of BA: the embryonic and the perinatal type. The embryonic form, which makes up 20% of the patients, is associated with other malformations, such as polysplenia syndrome, cardiac anomalies and annular pancreas (3). The perinatal form accounts for 80% of cases of BA, and it is the main focus of this thesis.

Surgery is the only curative option (4). In 1950s, Japanese surgeon Kasai developed the Kasai procedure (5). During this procedure the fibrotic hilar plate is resected and a Roux-en-Y portoenterostomy is constructed. The resection of the fibrotic hilar plate offers the possibility of restoring bile flow via small patent bile ducts present in the resection margin. Despite a successful Kasai procedure, two thirds of children of BA will develop progressive liver fibrosis, necessitating a liver transplantation later in life (6). From 1968 to 1999, 2554 pediatric patients receiving liver transplant have been registered by European Liver Transplant Registry (ELTR). BA is the most frequent indication for liver transplantation in children (7).

However, little is known about the etiology of this disease. Summarizing the possible pathogenetic mechanisms involved in the perinatal form of BA there are five possibly interrelated factors: 1) a defect in morphogenesis of the biliary tract, 2) a defect in fetal circulation, 3) environmental toxin exposure, 4) viral infection, 5) immunologic dysregulation (8).

This thesis is divided in two parts, focusing on: I) basic research into the etiology of BA using the rhesus rotavirus (RRV)-induced murine BA model; and II) clinical aspects of human BA and other cholestatic liver diseases in children.

Part I: Different aspects in the development of biliary atresia in an animal model.

As BA is a rare disease, the development of a valid animal model, which can reproduce the clinical and histomorphologic changes of human BA over time, can add to a better understanding of this disease. Over the last years, a murine model induced by intraperitoneal injection of RRV has been established (9). In this animal model, a gradually developing yet complete and irreversible obstruction of the extrahepatic bile duct can be induced in newborn mice without surgical manipulation. The proposed mechanism of this model is an infection of the biliary epithelium by the virus followed by a secondary (auto)immune mediated inflammatory biliary obliteration (see figure 1) (10). Furthermore, the clinical picture, with progressive cholestasis and subsequent hepatic changes, is similar to that observed in human BA. The RRV model has, therefore, become an accepted model for human BA. A systematic review on the studies of this animal model is provided in **Chapter2**.

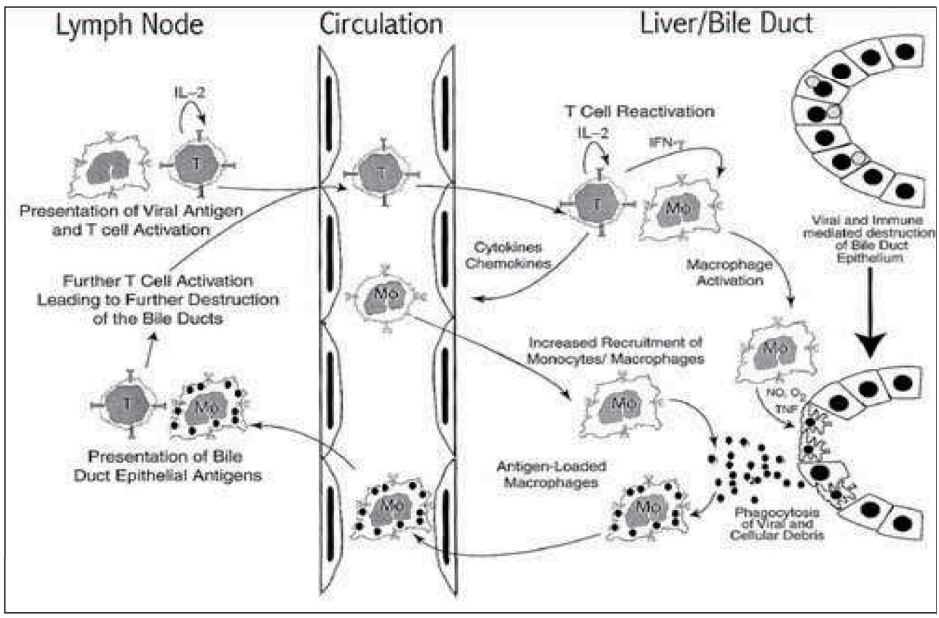

Figure1. Proposed mechanism of murine BA (11) .

The role of the Hedgehog signal transduction pathway and epithelial to mesenchymal transition in the development of murine biliary atresia

The Hedgehog (Hh) signaling pathway, one of the most important morphogenic pathways, is highly involved in embryogenesis. It is crucial for the early development of the heart, lung, gut and nervous system (12).While Hh signaling is virtually absent in the normal liver, it has recently been demonstrated that the Hh pathway is involved in the regulation of remodeling responses to biliary obstruction. Hh controls tissue (re)construction by regulating the viability and migratory activity of various types of Hh-responsive cells, including the activation of the progenitor cell compartment in the liver (13). During biliary fibrosis there is also an increased paracrine Hh signaling between cholangiocytes and myofibroblasts, contributing to the development of epithelial-mesenchymal transition (EMT). Currently, growing evidence supports an important role for EMT in liver repair (14).
In the murine model of BA, RRV induces inflammation of the bile ducts followed by progressive biliary obstruction with cholestasis and liver injury. Little is known about the involvement of Hh signaling and EMT in the fibro-proliferative response in this model. This is investigated in **Chapter 3**. This might be a starting point for further investigations on the mechanism of progressive liver fibrosis in BA.

The role of inflammation mediated down-regulation of hepatobiliary transporters in the development of intrahepatic cholestasis and liver damage in murine biliary atresia

Bile acid feedback regulation provides a crucial mechanism in the regulation of bile acid homeostasis and to prevent hepatic bile acid toxicity during cholestasis (15). A schematic picture of basolateral and canalicular transporters, as localized in the hepatocyte, is given in Figure 2. However, bile acid regulation differs in various situations. During the development of murine BA, RRV induced intra- and extra-hepatic inflammation of the bile ducts is followed by a progressive inflammatory extra-hepatic bile duct obstruction (16). We hypothesized that early downregulation of hepatobiliary transport due to inflammation might lead to intrahepatic cholestasis even before obstruction of the extra-hepatic bile duct occurs, thus contributing to liver damage in the early stages of the disease. This hypothesis is investigated in **Chapter4.**

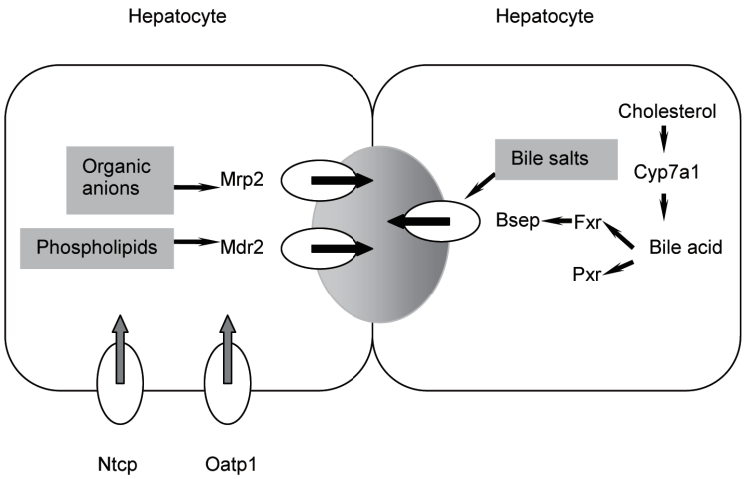

Figure2: Hepatobiliary transporters in the hepatocytes.

Part II: Clinical aspects of human biliary atresia and other pediatric cholestatic liver diseases.

We also investigated several clinical aspects of BA as well as outcome of surgical treatment of two other cholestatic liver diseases in children: progressive familial intrahepatic cholestasis (PFIC) and Alagille's disease. Three types of PFIC have been characterized, all caused by mutation of genes encoding for different hepatobiliary transporters proteins, such as bile salt export pump (BSEP, ABCB11) or multidrug resistance protein 3 (MDR3, ABCB4). Alagille syndrome is a genetic disorder that affects the liver, the heart, bone tissue and other systems of the body. Both PFIC and Alagille's disease are characterized by progressive cholestasis starting early in the first year of life. PFIC often progresses to cirrhosis and subsequent hepatic failure before the end of the second decade, while Alagille's disease sometimes follows more benign course. Partial external biliary diversion (PEBD) is a promising treatment for these diseases. It might decrease the bile salt pool size by partially diverting bile from the gallbladder through a loop of jejunum connecting the gallbladder with the abdominal skin (17). However, little is known about long term outcome after PEBD. A retrospective chart review was performed of all patients undergoing PEBD in the University Medical Center of Groningen (UMCG) between 2002 and 2005, focusing on the transplantation-free survival. The results are described in **Chapter 5.**

Despite a technically successful Kasai operation, long-term survival with native liver is only around 20% to 30%, even in experienced centers (18). Postoperative treatment with steroids might improve the prognosis by decreasing the incidence of cholangitis and the incidence of recurrent fibrosis at the portoenterostomy site (19). A systematic review and subsequent meta-analysis of the outcomes of BA patients who underwent a Kasai portoenteromy, comparing short and long term outcomes between patients who received steroids treatment postoperatively and patients who did not is performed in **Chapter 6**.

So far, several prognostic factors associated with a favourable outcome after Kasai portoenterostomy have been suggested, such as age at the time of the Kasai procedure, the degree of fibrosis and the presence and diameter of bile ducts in the resected hilar plate (20-23). As adequate surgical restoration of bile flow is widely thought to be the key to the success of the Kasai procedure, the prognostic significance of macroscopically visible biliary discharge as a prognostic factor in patients undergoing Kasai portoenterostomy for BA is determined in **Chapter 7**.

Finally, the results described in this thesis are summarized and future perspectives are discussed in **Chapter 8**.

Aims of this thesis:

1 To investigate Hedgehog signal transduction pathway activation and epithelial-mesenchymal transition in the development of murine biliary atresia;

2 To investigate the hepatobiliary transport regulation in the development of murine biliary atresia;

3 To investigate the clinical aspects, including outcomes and prognosis of surgical treatment and postoperative treatment, on human biliary atresia and other pediatric cholestatic diseases.

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PART I

Different aspects in the development of biliary atresia in an animal model

CHAPTER 2

The rota–virus induced murine biliary atresia model: current concepts and new areas of investigation

Huiqi Yang, Jan B. F. Hulscher, Henkjan J. Verkade, Robert J. Porte

Abstract

Biliary atresia is a disease of infancy of unknown origin in which predominantly the extrahepatic bile ducts obliterate. Despite a technically successful Kasai operation, liver damage still tends to progress. Survival with native liver is only around 20% to 30% at 10-year follow-up, even in experienced centres. Over the last decade, remarkable progress has been made regarding the establishment of an animal model for a better understanding of the pathogenesis of this disease. The rotavirus-induced murine model of biliary atresia which reproduces the key clinical and histomorphologic features of human BA is considered to be a valid animal model for investigating perinatal form of human BA. This model has been extensively studied on immunological aspects. The mechanism of this model is a primary viral infection targeting the biliary epithelium followed by secondary immune and autoimmune mediated biliary obliteration. There is a prominent Th1 response in the early phases of BA. CD8+ T cells contribute to the activation of inflammatory net work and development of the phenotype of BA. Among Th1 inflammatory cytokines, inactivation of *Ifn-g* can prevent biliary obstruction but not initiation of inflammation. Apoptosis activated by *Tnf-a* and *Ifn-g* seems to be the direct mechanism to result in bile duct injury. However, in order to provide more information for exploring clinical solution, more researches on the liver fibro-proliferative response and the impact on liver function after rota-virus infection are needed.

Introduction

Biliary atresia (BA) is a disease of infancy of unknown origin in which predominantly the extrahepatic bile ducts obliterate (1). Bile flow can be re-established in up to 60-80% patients by performing a Kasai portoenterostomy in which the fibrotic hilar plate is resected and a Roux-Y portoenterostomy is performed. Despite a technically successful Kasai operation, liver damage still tends to progress. Survival with native liver is only around 20% to 30% at 10-year follow-up, even in experienced centres (2).

Although there are a few theories about the etiology of the disease, its cause is as yet unknown. Possible mechanisms involved are (3): 1) a defect in morphogenesis of the biliary tract, 2) a defect in fetal circulation, 3) environmental toxin exposure, 4) viral infection, 5) immunologic dysregulation.

As this is a rare disease and human material is relatively limited, the development of a valid animal model, which can reproduce the clinical and histomorphologic changes of human BA over time, can add to a better understanding of this disease. Over the last decade, remarkable progress has been made regarding the establishment of such an animal model.

The development of an animal model of BA

Although some animal models, for example reovirus type3-induced BA (4) demonstrate a fibrosing inflammation of the bile ducts, no complete and definitive atresia can be developed. However, this can be induced by other two animal models. In the model of common bile duct ligation (5, 6), many features in patients with BA are observed, such as ductular proliferation, inflammatory infiltrates, portal and periportal fibrosis in the liver. Even though the expected consequences of mechanically induced cholestasis can be demonstrated in this model, the pathogenesis of BA such as virus infection and subsequent inflammatory obstruction can not be mimicked.

1. Rotavirus- induced murine model

Over the last decade, the rotavirus-induced murine model of biliary atresia has been extensively studied. In 1993, Riepenhoff-Talty et al (8) first described that orally administered Group A rotavirus leads to temporary and incomplete extrahepatic biliary obstruction in mice. Histological changes like fibrosis and ductular proliferation similar to those of human BA were found in the liver, however, no obvious atresia as described in human was observed.

Afterwards, a murine model induced by intraperitoneal injection of Rhesus Rotavirus (RRV) was established (9-11). EHBA with complete and irreversible obstruction of extrahepatic bile duct without direct manipulation can be developed progressively in newborn mice in this model. Furthermore, the clinical course with progressive biliary obstruction and hepatic changes leading to fibrosis is similar to human BA.

2. Symptoms and histopathologic changes

After intraperitoneal injection, mice usually become jaundiced after one week. Complete biliary obstruction develops after two weeks. Animals become profoundly icteric, have an oily fur and produce alcholic stools. In the blood, an increase in enzymes associated with a deteriorated liver function is observed. Serum bilirubin begins to increase at 1-week and remains at high level afterwards. Histology demonstrates an edematous swelling of the entire extrahepatic bile duct at seven days after infection (9). At the same time, a severe inflammation of small and large intrahepatic and extrahepatic bile ducts can be observed. Infiltration with neutrophils and mononuclear cells and epithelial destruction are found. At 14 days after inoculation, the inflammation of intra- and extrahepatic duct tends to regress. From this time on strictures can be found all along the extra-hepatic bile duct, sometimes associated with pre-stenotic dilatations or hydrops of the gallbladder. Animals eventually die at four weeks. This course of disease is very similar to the clinical picture of human BA.

A comparison between human and RRV-induced murine BA has been made by computerized three-dimensional reconstruction to systematically investigate the morphology of the intrahepatic and extrahepatic bile ducts (12). Despite of some differences, such as the cystic dilation of segments of the extrahepatic bile duct and the gallbladder which is more prominent in the murine model than in human BA, this

model demonstrates the involvement of both intrahepatic and extrahepatic biliary system, and is similar to the perinatal form of human BA.

Pathobiology

Is the cholangiocyte the primary target of RRV?

RRV targets cholangiocytes rather than hepatocytes. This is determined by double staining of cytokeratin-7 (biliary epithelium marker) and RRV. From five days after inoculation, colocalization occurs in both intra- and extra-hepatic biliary tract (13, 14).

Virus strain

To determine whether this tropism is unique for RRV, the differences between RRV and reovirus type 3 infection is compared (15). Biliary obstruction can not be demonstrated after reovirus type 3 infection, neither macroscopically nor microscopically. This elicits the question why different hepatotrophic viruses cause various changes in murine biliary tract.

The impact of four different strains of rotavirus, including SA11-FM, SA11-SM, EDIM and Wa, is tested (13). Only RRV and SA11-FM can trigger the clinical manifestations of biliary obstruction and subsequent mortality by targeting on biliary epithelium. Even though SA11-SM can lead to the clinical signs of hepatobiliary disease, mortality is remarkably lower than that of RRV and SA11-FM. SA11-SM is only identified in hepatocytes rather than biliary epithelium. These findings suggest that only specific strains of rotavirus target the cholangiocytes in a way that might eventually lead to the development of BA.

Cell surface intergrins determine the susceptibility of cholangiocytes to RRV

Viral attachment to and entry into the cells are considered to be two separate but complimentary steps in the viral infectious cycle. Attachment of rotavirus to cells is regulated by the cell-surface expression of the integrins $\alpha_2\beta_1$, $\alpha_4\beta_{1,}$, $\alpha_V\beta_3$, $\alpha_X\beta_2$, which function as virus receptors (16-20). $\alpha_2\beta_1$ integrin is uniquely expressed on cholangiocytes compared with hepatocytes. Mice pre-treated by antibody against

$\alpha_2\beta_1$ demonstrate milder symptoms, and improved survival when infected with RRV. RRV titres in the extrahepatic bile duct from $\alpha_2\beta_1$ treated mice are also reduced after RRV challenge. The cell surface expression of the integrin $\alpha_2\beta_1$ therefore is a crucial determinant governing cholangiocyte vulnerability to RRV.

Timing of infection

In this model, a temporary immunological gap after birth is permissive for the induction of BA (21), and the highest incidence of cholestasis occurs when mice are inoculated 12-18 hours after birth. The later newborn mice are infected, the less likelihood of triggering BA. Adult mice do not develop the clinical process of BA by RRV infection, and infection of pregnant mice does not elicit clinical and histological changes of BA in their offspring. It might be speculated that the time window for inducing EHBA by the required amount of RRV meets an immunological gap during the newborn period of the Balb/c mice.

Virus titre

The induction of this model is related to the titre of RRV. Higher titres result in a higher incidence of BA (10^7pfu/ml leading to 100% BA, and 10^6pfu/ml resulting in 86% BA when virus infection within 12 hours after birth) Lower doses of RRV (10^3 or 10^4 pfu/ml) do not induce any sign of cholestasis. However, the titre higher than 10^6pfu/ml leads to a very aggressive course of the disease, and this effect rather than hepatotropic effect results in early lethality. For instance, infection with 10^7pfu/ml causes 100% lethality within nine days infection, and only extrahepatic inflammation but no atresia is visible at that time. Therefore, currently the common titre adopted for the murine BA model is 10^6pfu/ml.

After RRV infection a small percentage of mice (around 30%) do not develop any signs of BA, even though they are infected with the same dose of virus as the mice which develop BA (9, 21). Our group finds that only very low expression of Th1 inflammatory cytokines like *Ifn-g* and *Tnf-a* can be detected by QPCR in non-infected mice. Technical problems such as leaking of the solution with virus while infecting might be the cause. This coincides with the conclusions which from the

previously mentioned studies. Finally, dams who have been infected accidentally with rotavirus might also offer protection to the pups (21). This is currently under research.

What happens to the cholangiocyte after infection?

Apoptosis is a significant mechanism of biliary epithelium injury

Six days after RRV infection, apoptosis can be observed in portal tracts and in the extrahepatic bile duct. At the same time, the expression of apoptosis related genes like caspases1 and 4 and Granzymes A and B increases in the extrahepatic bile duct.

Activated Nuclear factor-kB (NF-kB) proteins (transcription factors that control important biological processes such as activation of inflammatory and innate immune response to viral infection but also apoptosis) are detected on the cholangiocytes of mice infected by RRV at five days and peaked at ten days, while weaker NF-kB immunoreactivity can be found until 15 days on mice injected by both RRV and NF-kB inhibitor pyrolidine dithocarbamate (PDTC) (22). There is no cholestasis and occlusion of EHBD in the latter group. In both livers and EHBDs of this group, weaker inflammatory reaction occurs compared to the mice infected by RRV, while the virus still can be demonstrated. Therefore, NF-kB at least partially mediates RRV-induced murine BA, but it does not affect the viral clearance.

Mitogen Associated Protein Kinase (MAPK) signalling contributes to cholangiocytes response after viral infection

The activation or inhibition of numerous downstream targets by MAPKs, like p38, extracellular signal regulated kinase (ERK1/2) and c-Jun NH_2-terminal kinase (JNK1/2), has been shown to be important in cell differentiation and growth. They are also involved in the induction of apoptotic cell death (23, 24). Recently, MAPK signalling is found to be activated after RRV infection (25). This contributes to the viral replication after RRV attachment to the cholangiocyte surface and entry into the cholangiocyte. Among three important members of MAPK family, ERK1/2 and p38 seem to be the most important, as they participate both in viral replication and in cholangiocyte injury in vitro. In vitro, the viral replication significantly decreased after pre-treatment of ERK1/2 and p38 inhibitor, and cell injury was remarkably

reduced after pre-treatment of ERK1/2 inhibitor. Therefore, MAPK signalling might govern the response of cholangiocytes to RRV challenge.

Immunology

Th1 inflammatory cytokines

A Th1 pathway predominates one week after RRV infection in the livers of BA mice, with $CD4^+$ T cells producing *Ifn-g* and *Tnf*-a in the portal tracts. Two weeks after infection, the inflammatory pattern becomes more $CD8^+$ T cell and macrophage mediated. Despite clearance of the virus after two weeks, this immune response persists. In micro array studies the majority of genes with higher expression in BA mice encode pro-inflammatory cytokines related to the Th1 pathway, such as CCL2, CCL5, CCR5, and with lower expression of TIMD2, which regulates Th2 pathway.
Th1 cytokines also decrease the viability of cholangicytes via activation of apoptosis (26). *In vitro,* the viability of cholangicytes can be decreased significantly by incubating cholangiocytes with *Ifn-g* and *Tnf*-a, while the viability can be well maintained by incubating together with Mx1013, a pancapase inhibitor, suggesting that Inf-g and Tnf-a are involved in inducing apoptosis.

The contribution of Ifn-g

Ifn-g$^{-/-}$ mice develop the symptoms of extrahepatic biliary obstruction within one week. In contrast to WT Balb/c mice, symptoms begin to resolve at two weeks, followed by a complete resolution after three weeks. Therefore, the initiation of the process is independent of *Ifn-g*. Interestingly, the phenotype of BA can be re-induced by daily administration of recombinant *Ifn-g* from 24 hours after RRV inoculation. Therefore, *Ifn-g* is paramount in the further development but not the initiation of BA in this model.
Damage to the biliary tract can be prevented by prophylactic treatment with IFN-a. Jaundiced mice treated by *Ifn*-a daily for one week begin to recover, while progression to complete biliary obstruction is arrested (9). Infected and prophylactic *Ifn-a* treated dams provide protection from virus damage to their offspring. *Ifn* exerts its function via two types of receptors: type I is mainly activated by *Ifn-a,* while type

Ⅱ is activated by *Ifn-g*. *Ifn-a* seems to exert its beneficial effect via the type I receptor. For, the incidence of BA increases in the type I or type I and type Ⅱ receptor knockout mice after RRV infection when compared to type Ⅱ receptor knockout mice or control mice. When the type I and type I and type Ⅱ receptor knockout mice are treated by recombinant *Ifn-a*, the protective effect of *Ifn-a* diminishes. Even though the incidence of BA in type Ⅱ receptor knockout mice is less increased compared to that of the type I or type I and type Ⅱ receptor knockout mice, it remains increased significantly compared to that of the control mice. This increase is mediated by significantly decreased recovery rate of cholestatic mice. Therefore, *Ifn-g* seems involved in the recovery from cholestasis rather than contribution to the biliary obstruction. This finding contradicts the previously mentioned study in which *Ifn-g* is considered to regulate the development to biliary obstruction. The reason for this discrepancy needs to be further studied.

The role of other inflammatory cytokines

The role of some other Th1 cytokines, like *Tnf-a* and *Il-12*, are also studied(3, 27). After RRV infection *Il-12* knock out mice still demonstrate growth failure, jaundice, alcoholic stool and decreased survival similar to wild type mice. Likewise, *Tnf-a* knock out mice do not demonstrate less severe symptoms or improved survival. Inhibition of *Tnf-a* eight to ten days after RRV infection does not reduce the severity or the incidence of the BA. Therefore, even though these two cytokines are highly elevated during the process of BA, they do not play an obligate role in the disease progress. The severity of biliary obstruction and the incidence of BA can not be minimized by loss of *Tnf-a* and *IL-12*.

CD8 T cells also contribute to the pathogenesis of BA.

As previous studies uncover a cellular phenotype consistent with a prominent Th1 response in the early phases of BA (28, 29), a further investigation is on the role of two main T lymphocytes subtypes in the injury of bile duct and the development of BA (30). After loss of CD4+ T cells, jaundice is delayed but biliary injury, obliteration and progression to BA are not affected. In contrast, after depletion of CD8+ T cells, symptoms are short-lived with gradual recovery from jaundice, despite the inability

of viral clearance. No or only minor biliary injury is observed and the biliary tract remains patent. Pericholangitis still exists. CD8+ T cells can activate a broad inflammatory network like IL-2, IL-12p40 and TNF-a besides producing IFN-g. Therefore, CD8+ T cells are considered to play an essential role in the pathogenesis of BA: they activate the inflammatory network, injure the cholangiocytes and drive the phenotype of BA. IFN-g alone is not sufficient to induce biliary obstruction, even though it is previously determined to regulate biliary obstruction by inflammatory cells.

Humoral immunity

Immunoglobulin deposits (IgG) can be detected at periductal areas at two weeks of BA mice (14, 31). This is after the clearance of virus from the biliary epithelium. Therefore, it is likely that the immunoglobulin is reactive with biliary epithelium instead of virus particles. Furthermore, auto-antibodies reactive specially to multi-biliary epithelium proteins can be detected by western-blot in the sera of BA mice but not control mice (31).

Autoimmunity

Autoimmune-mediated inflammation and injury persists despite of the clearance of virus (32). In order to determine whether auto-reactive T cells and auto-antibodies are specific to biliary epithelium, hepatic or splenic T cells are transferred from RRV-induced BA mice into naïve syngeneic severe combined immunodeficiency (SCID) recipients (31). When hepatic or splenic T-cells are transferred from mice infected with RRV into naive severe combined immunodeficiency (SCID) mice, the SCID mice develop bile duct inflammation and increasing serum bilirubin levels, yet no complete biliary obstruction. In vitro, analyses of T cell antigen specific to bile duct epithelium demonstrates significant increases in *Ifn-g*-producing liver and spleen T cells from two weeks old BA mice in the presence of bile duct epithelial homogenate. Furthermore, the increase in the number of *Ifn-g*-producing T cells occurs in a dose –response fashion with increasing amount of bile duct epithelial antigen. RRV-primed CD8+ cells are found to recognize biliary epithelium and promote cholangiocyte cytolysis in vitro, whereas RRV-primed CD4+ cells and naïve CD8+ cells can not (30). In conclusion, cellular autoimmunity components

exist in murine BA. The progress to biliary obstruction is partially attributed to biliary epithelium-specific T cell-mediated autoimmunity.

Future perspectives

In the animal model, many studies have focussed on the pathogenesis of biliary atresia. This research is mainly focused on immunological aspects. As yet little is known about functional effects of the development of biliary atresia. There is also scanty data on the liver response to injury in this model. One of the main clinical problems is that BA often results in liver cirrhosis and the need for liver transplantation even after bile discharge has been re-established via a successful Kasai procedure. The present model might offer the opportunity to further elucidate these aspects.

Functional effects of RRV induced bile duct obstruction

Bile acid feedback regulation provides a crucial mechanism in the regulation of bile acid homeostasis and to prevent hepatic bile acid toxicity during cholestasis. Previous studies demonstrated different regulatory patterns of hepatobiliary transport between obstructive and inflammatory cholestatic liver injury. For example, in the well known bile duct ligation (BDL) model (obstructive cholestasis), there is down-regulation of basolateral transporters (*Ntcp*) mRNA, while canalicuar transporters (*Bsep)* mRNA expression is maintained. On the other hand, in the LPS-induced (inflammatory) cholestasis model, there is a marked down-regulation of basolateral transporters mRNA but also a down-regulation of canalicuar transporters mRNA expression. This leads to intrahepatic cholestasis. As the RRV induced biliary atresia model is also an inflammatory model, it might be speculated that inflammatory changes also influence hepatobiliary transport adding to the development of cholestasis via downregulation of bile transport systems in the hepatocytes and/or cholangiocytes. Further elucidation of these mechanisms may provide new insight in the development of BA, and may offer new therapeutic modalities aimed at optimizing bile flow in the early stages of disease or post-Kasai.

The liver response to injury

Cholestatic liver injury results in what has become known as the liver fibro-proliferative response, and might lead to subsequent liver fibrosis. In human biliary atresia, expanded portal tracts with bile duct proliferation, edema, inflammation and

fibrosis, combined with bile plugs are the hallmarks of the diagnosis. However, biopsies from patients with biliary atresia probably depict a later stage of the disease when compared to the changes observed in the mouse model. This early liver response to RRV infection has not been characterized and may offer further insight in the early development of the disease.

Epithelial-mesenchymal transition (EMT) has been demonstrated to be involved in the liver fibro-proliferative response in several liver injury models or liver diseases, such as the bile duct ligation model and human liver cirrhosis. During EMT epithelial cells (such as cholangiocytes) loose some of the epithelial characteristics such as cell-cell adhesion, and adapt a more mesenchymal phenotype. EMT is partly driven by Hedgehog signaling, an embryogenic pathway essential for development and regeneration. Hh pathway mediated epithelial-mesenchymal transition has been shown to be involved in the regulation of remodeling responses to cholestatic liver injury after biliary obstruction. Hh controls tissue (re)construction by regulating the viability and migratory activity of various types of Hh-responsive cells. However, little is known about the involvement of Hedgehog signalling and epithelial-mesenchymal transition in the fibro-proliferative response in the BA model.

Conclusions

1 RRV-induced murine animal BA is a valid animal model for investigating human BA, as it reproduces the key clinical and histomorphologic features of human BA.

2 The model is the result of a primary viral infection targeting the biliary epithelium followed by secondary immune and autoimmune mediated biliary obliteration.

3 There is a prominent Th1 response in the early phases of BA. CD8+ T cells contribute to the activation of inflammatory net work and development of the phenotype of BA. Among Th1 inflammatory cytokines, inactivation of *Ifn-g* can prevent biliary obstruction but not initiation of inflammation.

4 Apoptosis activated by *Tnf-a* and *Ifn-g* seems to be the direct mechanism to result in bile duct injury.

5 Little is known about the effects of RRV infection on liver function

6 The liver fibro-proliferative response should be characterized in more detail, as progressive fibrosis is one of the hallmarks of human biliary atresia.

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CHAPTER 3

Early development of liver fibrosis in biliary atresia: occurrence of epithelial to mesenchymal transition and hedgehog signal transduction

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In preparation

Abstract

Liver fibrosis is an early event in biliary atresia. The murine model of biliary atresia offers the possibility to investigate the development of biliary atresia at the earliest stages, and therefore to assess the development of fibrosis during this process.

Aim: To test the hypothesis that liver fibrosis occurs early during development of biliary atresia, and to demonstrate the presence of epithelial to mesenchymal transition and Hedgehog signalling during this process. In the rotavirus-induced murine model of biliary atresia, we assessed the development of fibrosis by determining markers of fibrosis (type I collagen, aSMA, Tgf-β1), markers of epithelial to mesenchymal (S100a4) and Hedgehog signalling (Ihh, Shh, Ptc, Gli-2) by qPCR and immunohistochemistry. Seven days after viral inoculation, while there was only inflammation but no bile duct obstruction, fibrosis could already be demonstrated by periportal accumulation of aSMA positive myofibroblast cells with increasing mRNA levels of aSMA, type I collagen and Tgf-β_1. At the same time, ductular cells started to express the epithelial-mesenchymal transition marker *S100a4*. Periportal accumulation of ductular cells expressing Indian Hedgehog (but not Sonic Hedgehog), the receptor Patched and the Hh target gene *Gli2* was found. After 14 days biliary stenoses were visible, which concurred with an increase in serum conjugated bilirubin, indicative for extrahepatic bile duct obstruction. The fibro-proliferative process subqequently progressed, accompanied by increased levels of the epithelial to mesenchymal and Hedgehog parameters. Liver injury, repair and fibrogenesis are early events in the development of murine biliary atresia, occurring before the actual development of bile duct obstruction. Epithelial to mesenchymal transition and Hedgehog signal transduction parallel this fibro-proliferative process. These data suggest a role for Hedgehog signalling and epithelial to mesenchymal transition in the development of fibrosis in biliary atresia.

Introduction

Biliary atresia (BA) is a disease characterized by early and often rapidly progressive fibrosis due to an inflammatory/immunologic response directed towards the cholangiocyte in combination with the deleterious effects of bile duct obstruction (1). Even after a successful porto-enterostomy many children develop progressive fibrosis and cirrhosis, eventually necessating liver transplantation (2).

The murine model for BA which demonstrates many key features of human BA offers the possibility to study the early development of BA and the liver response over time (3, 4). In this model, rota-virus (RRV) induced intra and extrahepatic inflammation of the bile ducts is followed by progressive inflammatory extrahepatic bile duct obstruction (5).This is different from mechanically induced biliary obstruction such as the in bile duct ligation model (BDL), in which the bile ducts are acutely obstructed by ligation and not gradually via inflammation. The murine model for BA provides a tool to study the role of fibrosis in the pathogenesis of BA.

Epithelial to mesenchymal transition (EMT), in which proliferating cholangicytes lose some of their epithelial phenotypic characteristics and acquire a more mesenchymal phenotype (6), plays an important role in tissue construction during the embryogenesis, and has recently been demonstrated in chronic liver injury, probably remodeling the tissue in biliary fibrosis (7, 8).

One of the most important regulators of the liver stem cell component and promoters of EMT is Hedgehog (Hh) signal transduction. Hh signalling is initiated when Hh ligands, such as Sonic Hedgehog (shh) or Indian Hedgehog (Ihh), which are soluble, lipid-modified morphogens, bind to their receptor Patched (*Ptc*), a membrane-spanning receptor on the surface of Hh-responsive cells. This interaction results in the release of *Ptc* co-receptor, Smoothened (*Smo*), from the inhibitory influence of *Ptc* (9). This leads to the activation of Hh- regulated trans-activating factors, including the Glioblastoma (*Gli*) family of transcription factors. While Hh signaling is virtually absent in the normal liver, the Hh pathway is involved in the regulation of remodeling responses to cholestatic liver injury after biliary obstruction (10).

Little is known about the development of fibrosis in the BA model. We hypothesized that fibrosis occurs relatively early in the course of disease. Our first aim was

therefore to assess the development of fibrosis in the BA model. Our second aim was to demonstrate the early presence of EMT and Hh signaling activation during development of BA.

Methods

The murine Rhesus Rotavirus induced model of BA

Adult, pregnant virus-free Balb/c mice were purchased from Harlan (The Netherlands) and were kept in a specific pathogen-free Individual Ventilated Cage system. Mice received care in compliance with the guidelines of the local animal ethics committee according to the Experiments on Animals Act (1996) issued by the Netherlands Ministry of Public Health, Welfare and Sports. Newborn pups were infected intraperitoneally with 20 µl $1*10^6$ plaque-forming units (PFU) of the MMU18006 strain of RRV, which was kindly provided by Professor Claus Petersen (Medical School Hannover, Germany) (11). A control group consisted of mice injected with 20 µl 0.9% saline.

Mice were originally planned to be sacrificed at seven, 14 and 21 days after RRV infection or saline injection. As the sick mice began to lose weight from 14 days of age we terminated the last groups of RRV- and control injected mice at 18 days instead of 21 days of age.

At these three time points, the gross (microscopic) appearance of liver, gall bladder and common bile duct was recorded and the organs were harvested and frozen into liquid nitrogen for RNA isolation or stored at -80°C for histological analysis.

Histology and Immunohistochemistry

Frozen sections of liver and bile duct (4-μm thickness) were stained with haematoxylin and eosin using standard histological techniques. Separate sections were incubated with primary antibodies, rabbit polyclonal cytokeratin19 (ck19) antibody, α-SMA antibody, type I collagen antibody, Gli2 antibody, Ptc antibody (Abcam, Cambridge, UK), S100a4 antibody (Dako, Denmark), goat polyclonal Ihh antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for one hour at room temperature. For ck19, α-SMA, Gli2, Ptc, type I collagen and S100a4 immunostaining, goat anti-rabbit IgG horseradish peroxidase (HRP)-labelled (Dako, Denmark) was used as a secondary antibody and rabbit anti goat IgG HRP-labelled

(Dako, Denmark) was used as a tertiary antibody. For Ihh immunostaining, rabbit anti-goat IgG HRP-labelled was used as a secondary antibody and goat anti-rabbit IgG HRP-labelled was used as a tertiary antibody.

For ki67/ck19 double staining, sections were first immuno-stained for ki67, using ki67 monoclonal rabbit antibody (Novus Biological, US), goat anti-rabbit IgG-HRP antibody as a secondary and rabbit anti-goat IgG-HRP antibody as a tertiary antibody. The staining reaction was developed with AEC (3-amino-9 ethyl-carbazole). Subsequently, the sections were incubated with ck19 antibody using goat anti-rabbit with an alkaline phosphatase label (Dako, Denmark) as secondary antibody. The staining reaction was developed with solution of fast blue.

Mouse skin was used as a positive control for ck19 and ki67 immunostaining. Mouse intestine was used as a positive control for Ihh, Gli2 and Ptc immunostaining. Mouse uterus was used as a positive control for α-SMA immunostaining. Mouse brain was used as a positive control for S100a4 immunostaining. BA mice livers exposed to 1%BSA/PBS instead of a primary antibody were used as negative controls.

Gene expression

Total RNA was extracted from frozen liver by RNeasy Mini Kit (Qiagen GmbH, Germany). Following incubation with RNase-free DNase I (Invitrogen Life Technologies), reverse transcription was performed with SuperScript II reverse transcriptase and oligo (dT) (Invitrogen Life Technologies). 5 µl cDNA (1:25 diluted) was subjected to real-time PCR using SYBR Green I as a double-strand DNA-specific binding dye to quantify the expression of target genes and β-actin house keeping gene. Primers (Table 1) were designed by using Primer Express Software (Applied Biosystem). Data were analyzed with Sequence Detector System 2.3 (Applied Biosystem). The levels of gene expression were calculated as a ratio to the house keeping gene β-actin. The levels of gene expression were normalized to that of the control group, and the level of control group was set as 100%.

Statistics

The differences of mean expression levels between healthy control and BA mice were compared using the Mann Whitney U test. A p-value <0.05 was considered

statistically significant. Statistics were performed using SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA).

Table 1. Sequences of primers, probes and PCR product sizes for amplified products.

Gene (Gene ID)	Primers	Annealing temperature	Product size
β-actin (Actb) (11461)	(F): 5'-AGCCATGTACGTAGCCATCCA (R): 5'-TCTCCGGAGTCCATCACAATG	59ºC	81bp
Gli2 (14633)	(F): 5'- TTCCATGAAGCTCGTCAAGGT (R): 5'- TGTTAGCAAGTAACTGAGGAGACTACAA	60ºC	81bp
Ihh (16147)	(F): 5'- CCAGCCTCCTGAGCCTTTG (R): 5'- AGTCCCAGGTAGTAGGGTCACATT	60ºC	74bp
Shh (20423)	(F): 5'-CCAAGCAACCTGCTGAAAGTCTAT (R): 5'-AATGCGGAGGTTTGCCTTCT	60ºC	77bp
Ptc (Ptch1) (19206)	(F): 5'- CTCCAAGTGTCGTCCGGTTT (R): 5'- TGTACTCCGAGTCGGAGGAATC	60ºC	80bp
ck19 (Krt19) (16669)	(F): 5'- AGAGCGTGATCAGCGGTTT (R): 5'- TACTCCTGGTTCTGGCGCTCTA	60ºC	72bp
Afp (11576)	(F): 5'- AGGAGGCTATGCATCACCAGTT (R): 5'- TGGAAGATGAATTTATCCTCAGAGAA	60ºC	79bp
a- SMA (Acta2) (11475)	(F): 5'- GAGAAAATGACCCAGATTATGTTTGA (R): 5'- GGACAGCACAGCCTGAATAGC	60ºC	74bp
Type I collagen (Col1a1)(12842)	(F): 5'- GGAGAGTACTGGATCGACCCTAAC (R): 5'-CTGACCTGTCTCCATGTTGCA	60ºC	77bp
Tgf-β1 (Tgfb1) (21803)	(F): 5'- GCTCTTGTGACAGCAAAGATAACAAA (R): 5'- AGGTCGCCCCGACGTTT	60ºC	72bp
S100a4 (20198)	(F): 5'-TTGAGGGCTGCCCAGATAAG (R): 5'-GCAAACTACACCCCAACACTTCA	60ºC	81bp
Bmp7 (12162)	(F): 5'-GCGCTGGCGTCTGTGTT (R): 5'-TTCATTCATGGGTTTTATTGTGACA	60ºC	73bp

Results

The development of biliary atresia in the animal model

To induce BA, we infected the newborn mice intraperitoneally with RRV. Sixty-five percent of infected animals developed the phenotype of BA characterized by jaundice, oily fur and high levels of urine bilirubin. Although inflammation of the bile ducts could be observed at 7 days, obstruction became apparent (microscopically) only at 14 days. The obstruction was paralleled by an increase in conjugated bilirubin (data not shown).

Injury and proliferation of cholangiocytes and hepatocytes

To assess the proliferative response of cholangiocytes after injury, single staining with ck19 and double staining with ck19/ki67 were used. In contrast to control mice, portal tracts in the liver of BA mice harboured more ductular cells, including increasing numbers of cells expressing ck19 as of seven days after infection Moreover, the livers of BA mice demonstrated pronounced cholangiocellular proliferation, including increasing numbers of ki67 (a marker of proliferation) / ck19 positive ductules and bile ducts in the portal tracts over time (Figure 1). The mRNA expression of ck19 (a marker of bile ductular cells) was significantly increased at 18 days, the mRNA expressions of ck19 was 7.8 fold higher respectively compared to the expression of control mice (Figure1).

In contrast to the early cholangiocellular proliferative response at seven days, the livers of BA mice did not show proliferation of hepatocytes until 18 days, including increasing number of ki67 positive hepatocytes (Figure 1).The mRNA expression of alpha-fetoprotein (*Afp*, marker of oval cells and fetal hepatocytes) increased significantly at 18 days compared to the expression of control mice (Figure 1). In BA mice, hematoxin-eosin staining demonstrated visible foci of parenchymal necrosis at 18 days (Figure 2).

Activation of stellate cells and progression of liver fibrosis

To demonstrate liver fibrosis, we compared the expression of the liver fibrosis markers *Tgf-β_1,* α-*SMA*, and type I collagen between control and BA mice.

Histologically, the livers of BA mice demonstrated progressive accumulation of *a-SMA-expressing* myofibroblastic cells, and progressive deposition of collagen in the portal area from seven days on. (Figure 3) The mRNA expression of *a-SMA* increased to 2.6 fold at 18 days on average compared with control mice. At 18 days, the mean mRNA expressions of *collagen1a (1)* and *Tgf-β1 w*ere 2.1 fold and 1.4 fold respectively in BA mice compared to control mice. (Figure3)

Epithelial to mesenchymal transition

To assess the presence of EMT, we used immunohistochemistry to demonstrate *S100a4* expressing cells and qPCR to determine the expression of *S100a4*.

Immunohistochemistry showed scattered *S100a4* expressing ductular cells and immune cells in the portal area from seven days on in BA mice (Figure 4). Consistent with the results of immunohistochemistry, mRNA expressions of *S100a4* were higher in BA mice when compared to the expression in control mice. *S100a4* expression increased over 14 fold at 18 days (Figure 4). The mRNA expressions of bone morphogenetic protein 7 (*Bmp7*) (inhibitor of EMT) were decreased in BA mice, while no significant difference between BA and control mice was found in the expression of the insulin-like growth factor I receptor 1(*Igf1r*) which promotes EMT (data not shown).

Hedgehog signal transduction

To assess the presence of Hh signalling, we used immunohistochemistry to demonstrate the Hh ligands *Ihh*, its receptor *Ptc,* the Hh target gene *Gli2* expressing cells, and qPCR determine expression of the Hh signalling related genes.

Immunohistochemistry demonstrated no hepatic expression of Hh ligands in control mice, but revealed increasing numbers of periportal *Ihh* positive cells from seven days on. Most of the *Ihh –expressing* cells were bile ductular cells, and some were stromal cells (Figure 5). In BA mice, some bile ductular cells and stromal cells also exhibited *Ptc* immunoreactivity from seven days, while no *Ptc* staining was found in the livers of control mice (Figure 5).

Consistent with the immunohistochemistry results, mRNA expression of *Ihh* increased over time. Induction of *Ptc* was more robust, resulting in an 11-fold higher

in mRNA expression levels in the livers of BA mice compared to control mice (Figure 6). In contrast, the mRNA expression of *Shh* was similar in BA and control mice. Immunohistochemistry also demonstrated increased staining for *Gli2* in bile ductular cells and stromal cells in portal areas from seven days on in BA mice, and some inflammatory cells were also *Gli2*-positive (Figure 7), even though the whole liver mRNA expression of *Gli2* was not increased in BA mice compared with the expression of control mice (data not shown).

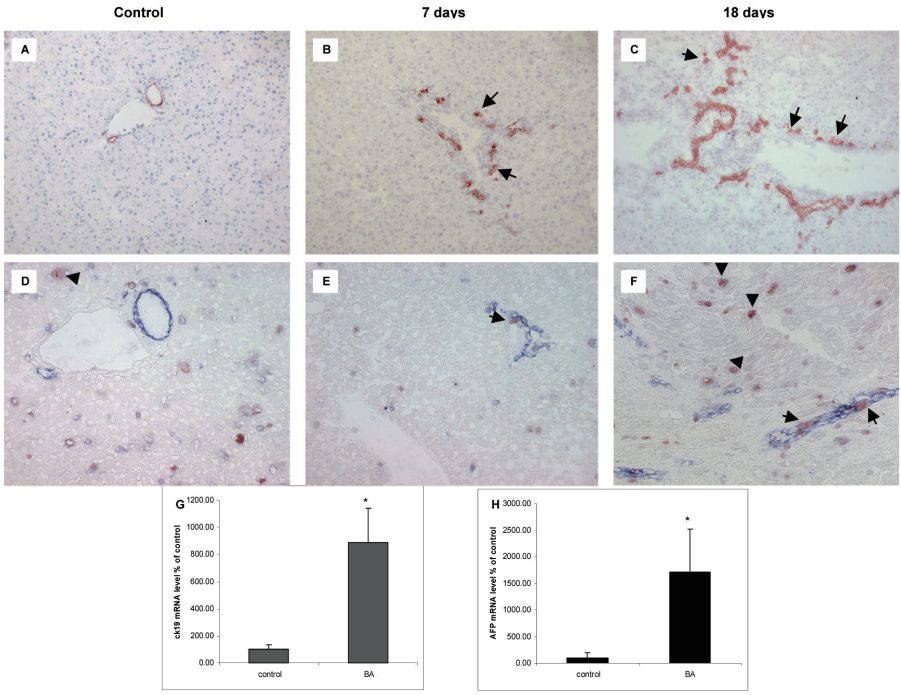

Figure 1. Cholangiocellular and hepatocellular proliferation.

Ck19 staining of a normal portal tract in control mice (A, magnification*200), and BA mice demonstrating ductular reaction (black arrow) at seven days (B, magnification*200), and 18 days (C, magnification*200).

Double staining with ck19 (blue, cytoplasmic staining) and ki67 (red, nuclear staining) demonstrates the absence of proliferation in the portal tracts in controls (D, magnification* 400). Ki 67 positive nuclei of ductules (black arrow) were demonstrated in BA mice at 14 days (E, magnification*400). Ki67 positive nuclei of bile ducts (black arrow) and hepatocytes (arrow head) were found in BA mice at 18 days (F, magnification*400).

The lower panel shows the mRNA levels of cholangiocyte markers, *ck19* (G), hepatocyte marker *Afp* (H) in BA and control mice at 18 days. The level of the control group was set as 100%. *) $p < 0.05$.

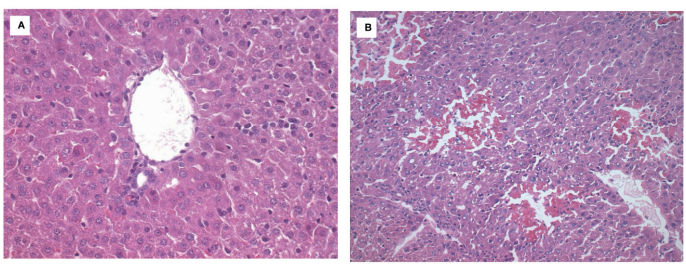

Figure 2. H&E staining showing normal portal tract morphology in control mice (A, magnification*400), and liver necrosis in BA mice at 18 days (B, magnification*200)

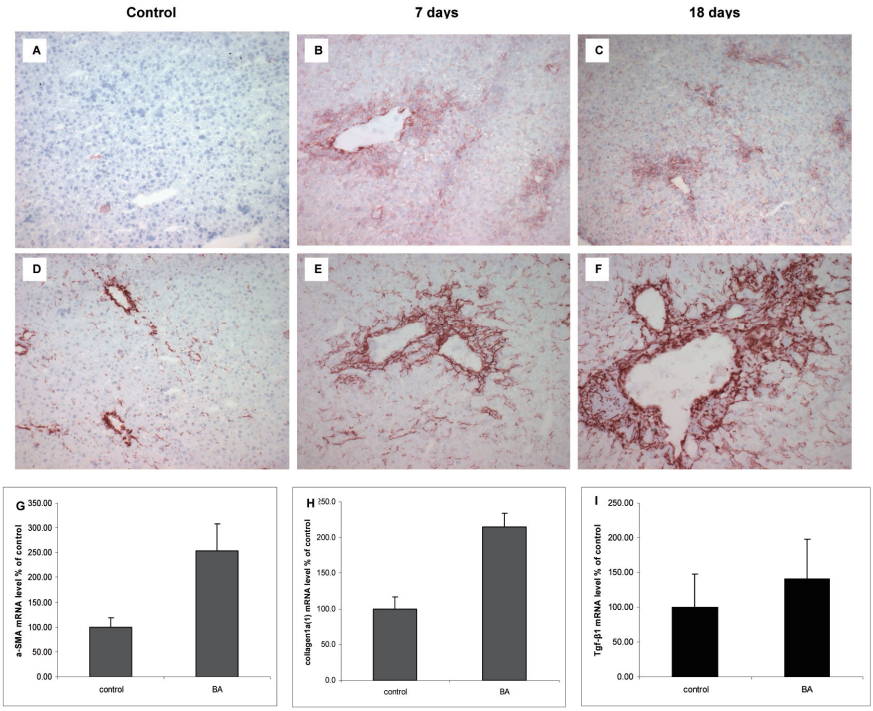

Figure 3. The development of liver fibrosis. Alpha-smooth muscle actin (a-SMA) staining showed normal portal tract in control mice (A, magnification*200) and periportal accumulation of a-SMA expressing myofibroblast cells at 14 days (B, magnification*200) and 18 days(C, magnification*200) in the livers of BA mice.

Type I collagen staining of a normal portal tract in control mice (D, magnification*200), while we observed increasing periportal deposition of collagen at 14 days (E, magnification*200) and at 18 days (F, magnification*200) in the livers of BA mice.

The lower panel shows the mRNA levels of *a-SMA* (G), collagen1a (1) (H) and transforming growth factor beta1 (*Tgf-β1)* (I) in BA and control mice at 18 days.

The level of the control group was set as 100%. *) *p*< 0.05.

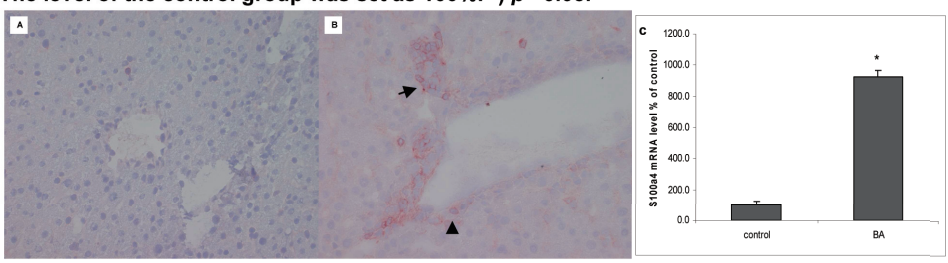

Figure 4. *S100a4* immunohistochemistry in the liver. Absence of *S100a4* staining in control mice (A, magnification*400), periportal accumulation of *S100a4-expressing* cells (B, magnification*400) at 14 days in BA mice. The mRNA levels of *S100a4* in BA and control mice at 18 days(C). The level of the control group was set as 100%. *) *p*< 0.05.

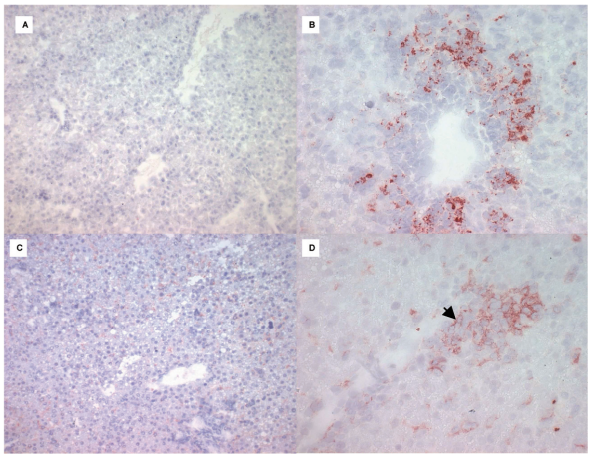

Figure 5. Indian hedgehog (*Ihh*) and Patched(*Ptc*) immunohistochemistry in the liver. Absence of *Ihh* staining in control mice (A, magnification*200), periportal accumulation of *Ihh-expressing* cells (B, magnification*400) in BA mice. Absence of *Ptc* staining in control mice (C, magnification*200), periportal accumulation of *Ptc-expressing* cells (D, 400*original magnification) in\BA mice.

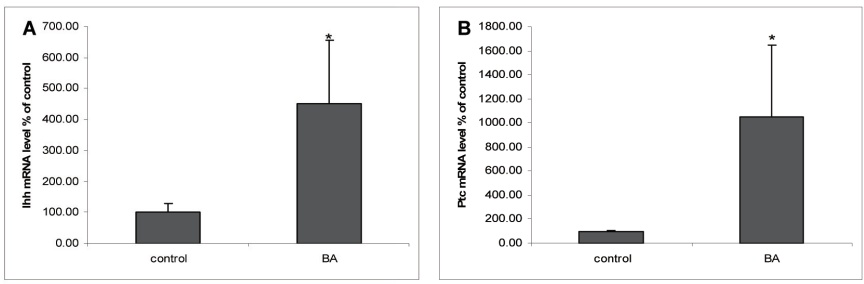

Figure 6. The mRNA levels of *Ihh* (A) and *Ptc* (B) in BA and control mice at 18 days. The level of the control group was set as 100%. *) $p < 0.05$.

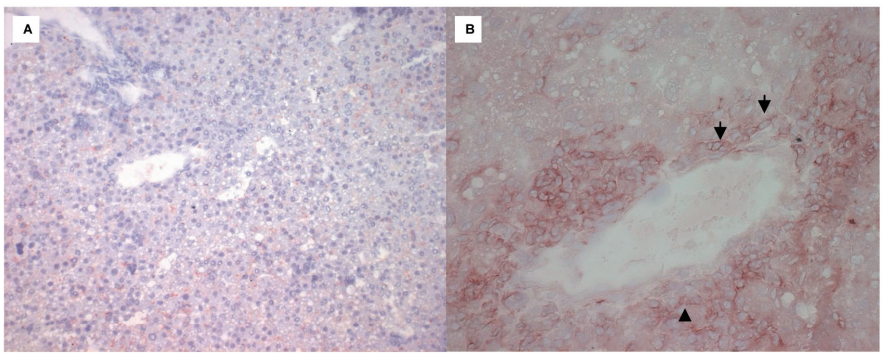

Figure 7. *Gli2* immunohistochemistry in the liver. Absence of *Gli2*staining in control mice (A, magnification*200), periportal accumulation of *Gli2-expressing* cells (B, 400*original magnification) in one week BA mice.

Discussion

We have demonstrated that the fibro-proliferative reaction occurs very early during the development of biliary atresia. In the present model, the fibro-proliferative process is initiated even before bile duct obstruction. Furthermore, EMT and Hh signalling also occur early in the development of the fibro-proliferative response. These data suggest a role for EMT and Hh in the liver fibrogenesis during the development of BA. According to our knowledge, the present study is the first to investigate the fibro-proliferative process in the RRV-induced murine model of BA.

Already at seven days, thus prior to biliary obstruction, we observed a ductular reaction with proliferation of cholangiocytes. Immunohistochemistry also demonstrated periportal accumulation of *a-SMA*-expressing stellate cells and increased deposition of collagen, indicative of developing liver fibrosis. Rotavirus elicits an inflammatory response and subsequent destruction of bile ducts. This is rapidly followed by proliferation of cholangiocytes (the ductular reaction), while concomitant activation of HSCs increases the extra-cellular matrix (12). Together this constitutes the progressive fibro-proliferative reaction, which thus starts early during the development of BA.

As we have described previously, the early development of intrahepatic cholestasis, which is the result of inflammation related down regulation of hepabiliary transporters at the early stages of BA could be another reason of the early occurrence of fibro-proliferative response (13, 14).

In contrast to the early cholangiocellular proliferation, hepatocellular damage was followed by proliferation of hepatocytes occurred only after 14 days. This relatively late hepatocellular effect might be due to biliary obstruction, and bile acid toxicity, but not to the primary infection and the immunological response directed at the cholangiocyte (15, 16). Taken together this response to RRV infection shows a different pattern from that observed in the bile duct ligation model (BDL), in which both hepatocellular and cholangiocellular proliferation occur within one week after BDL (17).

In the current study, we observed periportal *S100a4* expressing ductular cells already at 7 days. On the mRNA level, there was also an increase in *S100a4* with a decrease in EMT inhibitor *Bmp7* levels. The increase on the mRNA expression of

S100a4 was due to the *S100a4* expressing of both ductular cells and inflammatory cells (18). These results suggest the presence of EMT at an early stage in the development of BA. EMT, as an important mechanism for tissue morphogenesis in the fetal development and tumor metastasis (19), has been determined to have a certain role in the repair of injury in other tissues, like kidney (20). Furthermore, a previous study showed that EMT is likely to play role in the pathogenesis of biliary fibrosis (21).

In the present study the increase in *S100a4* mRNA levels paralleled that of *Ihh* and *Ptc*. In addition, *S100a4, Ptc* and *Gli-2* were present on ductular cells. These observations further suggest a close relation between EMT and Hh signalling, although a direct causal relation has not been proven in the present study. It has been known that Hh signalling regulates EMT during fetal development (22), while now growing evidence also supports a role for EMT regulated by Hh signalling in tissue remodelling after liver injury (23, 24). In livers of patients with biliary cirrhosis, mesenchymal markers and the Hh signalling trans-activating factor gene *Gli2* co-localize on cholangiocytes. This was also observed on cholangiocytes isolated from BDL-induced biliary cirrhosis in rats. Co-culturing these cholangiocytes with Hh-producing HSCs promotes EMT, whereas this effect could be blocked using Hh-antibody. Altogether these data suggest that Hh signalling actually promotes EMT.

We demonstrated that ductular and stromal cells expressing the Hh ligand *Ihh,* its receptor *Ptc*, and the target gene *Gli2* accumulated in the portal tracts, paralleling the appearance of activated HSCs. So far, the relation between Hh and HSCs activation has not been determined in detail yet. Hh ligands are considered to be produced by activated HSCs and hepatic progenitor cells (25). *In vitro* activation of HSCs results in production of Hh ligands and further activation of this fibrogenic cell population. In addition, the accumulation of Hh ligand expressing cells is located in the portal area. This is also the location where most myofibroblast activation and liver damage occurs. This was also observed in the present study. Together this data suggests that Hh signalling is involved in the fibrogenic response.

We observed accumulation of Hh ligand and its receptor with a concomitant increased proliferation activity of ductular cells. Besides being involved in fibrosis, Hh signalling might promote the survival and accumulation of progenitor cells (26). Blockade of Hh activity decreases survival of progenitors, whereas stimulation of Hh activity inhibits apoptosis. In the BDL rat model(27), Hh-signalling is activated,

with expanding Hh-responsive ductular cells representing a potential liver progenitor cell population. After BDL in Ptc-deficient rats, the liver demonstrates an expanded and increased fibroductular response with poorly organized ductular structure. After reversal of cholestasis by performing a Roux en Y hepaticojejunostomy, the expression of Hh ligand decreases, and the number of Hh-responsive ductular cells decreases (27). In vitro, incubation of immature cholangiocytes with Hh ligands can inhibit the apoptosis of cholangiocytes. These data suggest that Hh ligand might promote the survival of progenitors by inhibition of apoptosis activity.

The advantage of the present animal model is the availability of specimens at the early stages of BA development. This model thus offers insight in the early development of biliary atresia and biliary atresia related fibrosis. The findings of the present study further emphasize the importance of early diagnosis and treatment of this disease with its early and often rapidly progressive fibrosis.

Conclusion

During the development of biliary atresia, the fibro-proliferative response occurs early, even before bile duct obstruction. This early fibro-proliferative response is associated with the beginning of epithelial to mesenchymal transition and hedgehog signalling activity. These findings open new avenues for further research in the pathogenesis of BA. These investigations should be aimed at elucidating the causal relations between Hedgehog signalling, epithelial to mesenchymal transition and the fibro-proliferative response in this model.

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CHAPTER 4

Inflammation mediated down–regulation of hepatobiliary transporters contributes to intrahepatic cholestasis and liver damage in murine biliary atresia

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Pediatric Research, in press

Abstract

To investigate the hypothesis that during the development of biliary atresia early changes in hepatobiliary transport are mainly related to the inflammatory process and lead to intrahepatic cholestasis and subsequent liver injury, livers from mice with rhesus rotavirus-induced biliary atresia were analyzed for mRNA expression of hepatobiliary transporters, nuclear receptors and inflammatory cytokines. Seven days after inoculation, despite high bile acid concentrations in the liver, gene expression of canalicular and basolateral hepatobiliary transporters and their regulatory nuclear receptors was down-regulated with concomitant increase in gene expression of inflammatory cytokines and rise in serum unconjugated bilirubin. At 14 days, hepatobiliary transporters and nuclear receptors remained downregulated although the inflammatory response subsided. The percentage of conjugated bilirubin started to increase as extrahepatic biliary obstruction occurred. At 18 days, expression of hepatobiliary transporters remained low, expression of nuclear receptors returned to normal, while expression of inflammatory cytokines decreased further. Moreover, histology demonstrated progressive inflammation, bile duct damage, ductular proliferation and hepatocyte necrosis. In conclusion, intrahepatic cholestasis due to inflammation-related down-regulation of basolateral and canalicular hepatobiliary transporters is an early event in the development of biliary atresia. Intrahepatic cholestasis contributes to the development of jaundice and liver injury.

Introduction

Changes in the expression of hepatobiliary transporters provide a crucial mechanism to regulate bile acid homeostasis and to prevent hepatic bile acid toxicity in hepatocytes during cholestasis (1-7). The basolateral transporters, sodium-taurocholate co-transporting polypeptide (Ntcp; Slc10a1) and organic anion-transporting polypeptide (Oatp; Sl21a) are the major transporters for the uptake of bile acids and organic solutes from blood into the hepatocyte.

The canalicular transporters Bile salt export pump (Bsep; Abcb11), Multidrug resistance-associated protein 2 (Mrp2; Abcc) and Multidrug resistance protein (Mdr2; Abcb4) are responsible for the excretion of bile salts, organic anions and phospholipids into bile.

The nuclear receptors Farnesoid X receptor (Fxr, NR1H4) and Pregnane X receptor (Pxr, NR1I2) also play an important role in the regulation of bile acid metabolism. High bile acid levels can activate FXR, which directly or indirectly induces Bsep expression.

Different forms of cholestasis may exhibit different transport regulatory mechanisms. Although bile duct ligation and Lipopolysaccharide (LPS) - induced cholestasis are two well studied cholestasis models, little is known about regulation of hepatobiliary transport during the development of biliary atresia (BA). BA is a progressive inflammatory fibro-obliteration of the extrahepatic biliary tree of the neonate of unknown etiology.

The Rhesus Rotavirus (RRV)-induced biliary atresia mouse model offers the possibility to longitudinally study both acute and chronic cholestatic responses. It demonstrates many key features of human biliary atresia (8, 9). In this model, RRV induced intra and extrahepatic inflammation of the bile ducts is followed by progressive inflammatory extrahepatic bile duct obstruction (10, 11) This is different from mechanically induced biliary obstruction like bile duct ligation models (BDL).

Although the RRV BA model is based on a pan-cholangitis, hepatobiliary transport may also be altered during the early development of biliary atresia as the result of the inflammation itself. In the present study, we hypothesized that early changes in hepatobiliary transport due to inflammation might lead to intrahepatic cholestasis

even before obstruction of the extrahepatic bile duct occurs, thus contributing to early liver damage.

Methods

Infection of newborn mice with Rhesus Rotavirus

Adult, pregnant virus-free Balb/c mice were purchased from Harlan (The Netherlands). Mice received care in compliance with the guidelines of the local animal ethics committee according to the Experiments on Animals Act (1996) issued by the Netherlands Ministry of Public Health, Welfare and Sports.

Within 18 hours after birth, the newborn pups were infected intraperitoneally with 20 µl $1*10^6$ plaque-forming units (PFU) of the MMU18006 strain of RRV, kindly provided by Professor Claus Petersen (Medical School Hannover, Germany). Mice in the control group were injected with 20 µl 0.9% saline. Infected mice which died within the first six days or that were not fed by their mothers after infection were excluded from the study. All mice were weighed daily and were examined for jaundice, acholic stools and urine bilirubin (using urinalysis reagent strips, Bayer Crop, USA). Mice were planned to be sacrificed at seven, 14 and 21 days after RRV infection or saline injection. As sick mice began to lose weight after 14 days we terminated the last group at 18 days of age instead of 21 days.

At these three time points, blood samples were collected by cardiac puncture for determination of serum liver enzymes. The gross appearance of liver, gall bladder and common bile duct was recorded and the organs were harvested and frozen into liquid nitrogen for RNA isolation or stored for histological analysis.

Liver function and bile acid measurement

Serum liver enzymes including alanine aminotransferase (ALAT), gamma-glutamyl transferase (γ-GT) and serum bilirubin were assessed using the Ektachem DT60 Π system and DTSC Π Module (Johnson & Johnson Clinical Diagonostics Inc., Rochester, NY). For the determination of bile acids, parts of the livers of mice sacrificed at the same time point were pooled and crunched in liquid nitrogen. They were transferred in a total volume of 1.2 ml of 0.9% (w/v) NaCl. 115 µL 10 M NaOH solution and 1.4 mL of methanol were added and this solution was incubated at 80

˚C for 2 hours. After cooling to room temperature, 25 mL of water was added. After mixing and centrifuging 10 minutes at 2500 rpm, 100 µL of supernatant was measured enzymatically (12).

Histology and Immunohistochemistry

Frozen sections of liver and bile duct (4-*µ*m thickness) were stained with haematoxylin and eosin. Sections were also immuno-stained for cytokeratin 19, using Cytokeratin19 polyclonal rabbit antibody (Abcam, Cambridge, UK). Horseradish peroxidase (HRP)-labeled Goat anti-rabbit IgG (Dako, Denmark) was used as a secondary antibody,. HRP-labeled rabbit anti goat IgG (Dako, Denmark) was used as a tertiary antibody.

For ki67/cytokeratin 19 double staining, sections were immuno-stained for ki67, using ki67 monoclonal rabbit antibody (Novus Biological, US) as a first, Goat anti-rabbit IgG-HRP antibody (Dako, Denmark) as a secondary and rabbit anti goat IgG-HRP antibody (Dako, Denmark) as a tertiary antibody. The staining reaction was developed with AEC (3-amino-9 ethyl-carbazole). Afterwards, sections were incubated with cytokeratin19 antibody (polyclonal rabbit antibody, Abcam, Cambridge, UK) using goat anti-rabbit with an alkaline phosphatase label (Dako, Denmark) as secondary antibody. The staining reaction was developed with solution of fast blue.

Mouse skin was used as a positive control, RRV infected mice liver exposed to 1%BSA/PBS instead of a primary antibody was used as negative control.

All histological studies were assessed by one pathologist (A.S.H.G.) who was unaware of other study data.

Gene expression

Total RNA was extracted from frozen liver by RNeasy Mini Kit (Qiagen GmbH, Germany). Following incubation with RNase-free DNase I (Invitrogen Life Technologies), reverse transcription was performed with SuperScript II reverse transcriptase and oligo (dT) (Invitrogen Life Technologies CITY). 3 µl cDNA (1:20 diluted) was subjected to real-time PCR using the Taqman system in a ABIPRISM 7900HT Sequence Detector System (Applied Biosystem, Foster City, CA) to quantify expression of target genes. PCR amplifications were performed with

specific primers and probes (Table 1). Primers were designed by using Primer Express Software (Applied Biosystem). Data were analyzed with Sequence Detector System 2.3 (Applied Biosystem). The levels of gene expression were calculated as a ratio to the house keeping gene β-actin.

Membrane Isolation and Western Blotting

For isolation of total liver membranes, liver tissue was homogenized in 1 mM $NaHCO_3$ (pH 7.4), containing Complete protease inhibitor cocktail (Roche, Almere, the Netherlands). Homogenates were gauze-filtered and total membranes were isolated by centrifugation at 100.000 g for 1 hr at 4°C. Membrane pellets were re-suspended in 10 mM Tris/250 mM sucrose, pH 7.4, containing complete protease inhibitor cocktail and stored at -80°C. Protein was determined according to Lowry *et al* (13).

Total liver membranes were electrophoresed through polyacrylamide gels (7% for Ntcp; 5% for Bsep). Proteins were blotted onto a nitrocellulose filter (Amersham, Little Chalfont, UK) by tank blotting. Ponceau S staining was performed to check for equal protein transfer. Filters were blocked in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 4 % skim milk powder. Blots were incubated with polyclonal primary antibodies against *Ntcp* (14) or *Bsep* (15). After washing, immunocomplexes were detected using horseradish peroxidase conjugated donkey anti-rabbit IgG and Supersignal west pico chemiluminescent substrate (Thermo Scientific, Etten-Leur, The Netherlands). Band-densities were determined by using a Gel Doc XR (Biorad, Hercules CA, USA).

Due to the limited amount of tissue available and the large amount of tissue needed for Western blots only Ntcp and Bsep were studied at 14 days. This time point was chosen as it demonstrated the most significant down-regulation of mRNA expression.

Statistics

The differences of mean expression levels between control and rotavirus-infected groups were compared per time point using independent-samples *t* test or Mann Whitney U test as appropriate. A p-value <0.05 was considered to be statistically

significant. Statistics were performed using SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Rotavirus infection results in biliary atresia

To induce murine BA, we infected the newborn mice intraperitoneally with RRV. One mouse died three days after infection and was excluded. Sixty-five percent of infected animals developed the phenotype of biliary atresia. Seven days after RRV infection, jaundice became apparent in affected mice, and elevated urine bilirubin levels could be detected. In affected mice stenoses with or without prestenotic dilatations were visible in different parts of the biliary tree from 14 days onwards, which is in accordance with the literature (16).

Viral infection and inflammatory response

To investigate the viral infection and inflammatory response, mRNA levels of RRV proteins and inflammatory cytokines were quantified by qPCR. In control mice, viral proteins were not detectable. In BA mice, expression of the RRV non-structural protein *Nsp3* peaked at seven days, dropped at 14 days and was undetectable at 18 days (Fig 1). Similarly high levels of RRV structural protein (*Vp6*) were detected at seven days in BA mice which were not detectable at 14 days (data not shown).
Expression of *Ifng* and *Tnfa* remained low in control mice. Their expression in BA mice was substantially higher than that of control mice at seven days. *Ifn*g and *Tnf*a levels decreased rapidly at 14 days to return to normal at 18 days. In healthy control mice, the expression of *Il6* remained low, while its expression in BA mice was significantly higher than the expression of control group at 14 days, and decreased again at 18 days (Fig 2).

Cholestasis and liver functions

To determine the reason for cholestasis and the extent of liver damage, serum bilirubin, serum liver enzymes and bile salts in the liver were measured. In mice with BA high levels of serum bilirubin were observed. At one week the high level of

serum bilirubin was predominantly composed of unconjugated bilirubin. At 14 and 18 days the increase in serum bilirubin was predominantly due to conjugated bilirubin (Figure 2), which increased more than five fold compared to levels at seven

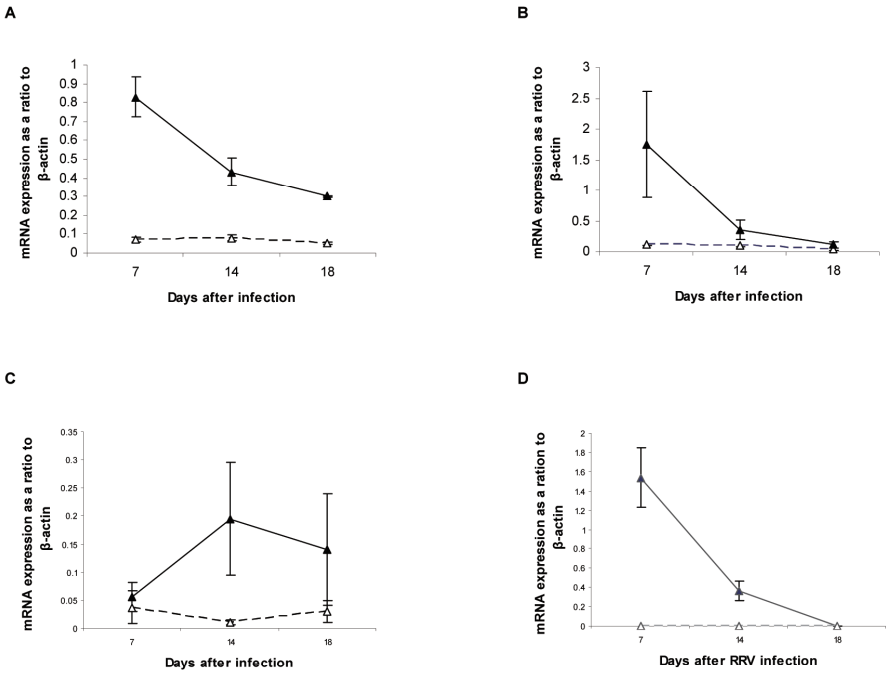

Figure 1. mRNA expression of Tnfa (A) and Ifng (B), Il6(C) and mRNA expression of encoding RRV non-structural protein (Nsp3) (D) in BA mice (closed triangles) and control mice (open triangles) at seven days, 14 days and 18 days.

days. Bile salts levels in the liver were already profoundly higher in infected mice at seven days, even before direct hyperbilirubinemia developed. ALAT, as a marker of liver damage, increased significantly compared to controls at 14 days and remained so over time (Figure2). γ-GT, as a marker of cholestasis and cholangiocyte injury, was undetectable in healthy controls, while its level was increasing over time in BA mice (Figure 3).

Liver histology

Hematoxin-eosin staining showed a progressive infiltration of the portal tracts with lymphocytes, neutrophils and macrophages from seven days on. (Figure 3) In addition, hepatic necrosis could be detected at 18 days. Cytokeratin 19 and

Cytokeratin19/ki67 double staining showed proliferation of ductular cells in both intra- and extrahepatic bile ducts from seven days on (Figure 4).

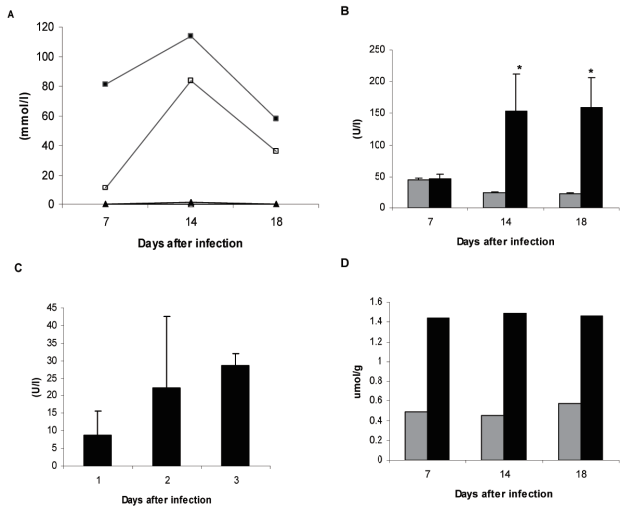

Figure 2. Serum bilirubin (A) in BA mice (total bilirubin: closed squares; direct bilirubin: open squares) and control mice (total bilirubin: closed triangles; direct bilirubin: open triangles); alanine aminotransferase (ALAT) (B), gamma-glutamyl transferase (γ-GT) levels (C), and mean liver bile acid concentration (D) in BA (black bars) and control mice (grey bars) at seven days, 14 days and 18 days. *) p< 0.05

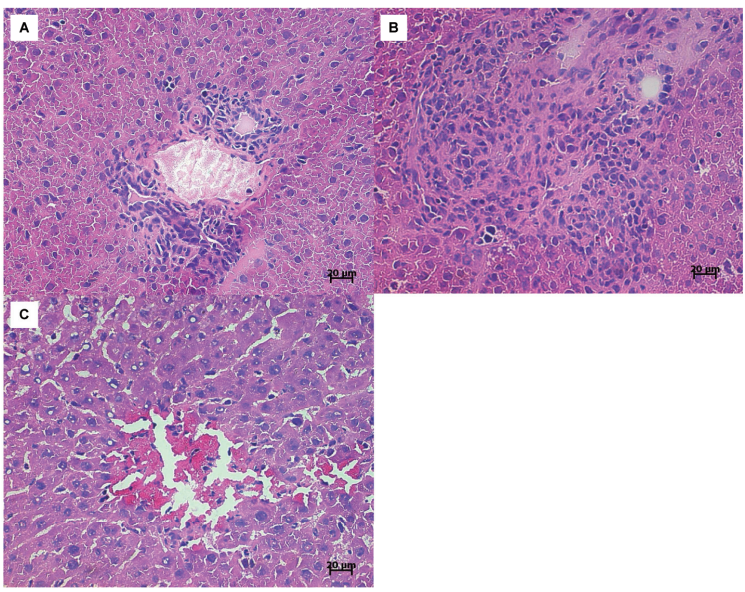

Figure 3. H&E staining showing normal portal tract morphology in control mice (A, magnification*400), inflammation of the portal tracts in BA mice at seven days (B, magnification*400) and liver necrosis in BA mice at 18 days (C, magnification*400).

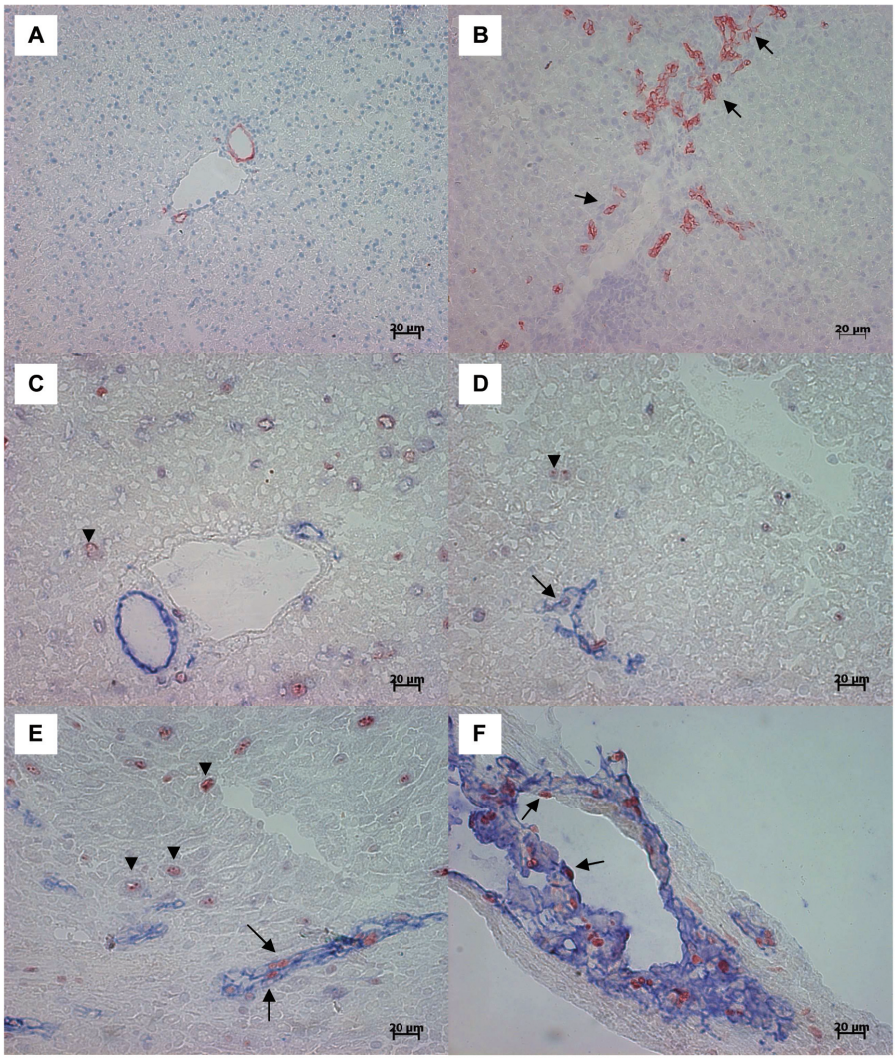

Figure 4. Ck19 staining of a normal portal tract (A, magnification*200), and BA mice demonstrating ductular reaction (black arrow) at 14 days (B, magnification*200). Double staining with ck19 (blue, cytoplasmic staining) and ki67 (red, nuclear staining) demonstrating the absence of proliferation in the portal tracts in controls, ki 67 positive hepatocytes were present (arrow head) (C, magnification * 400). Ki67 positive nuclei of ductules (black arrow) and ki 67 positive hepatocytes (arrow head) were demonstrated in BA mice at 14 days (D, magnification*400). Ki67 positive nuclei of bile ducts (black arrow) and hepatocytes (arrow head) were also found in BA mice at 18 days (E, magnification*400). Ki67 positive nuclei of the extrahepatic duct (black arrow) were observed at 18 (F, magnification * 400) days in BA mice.

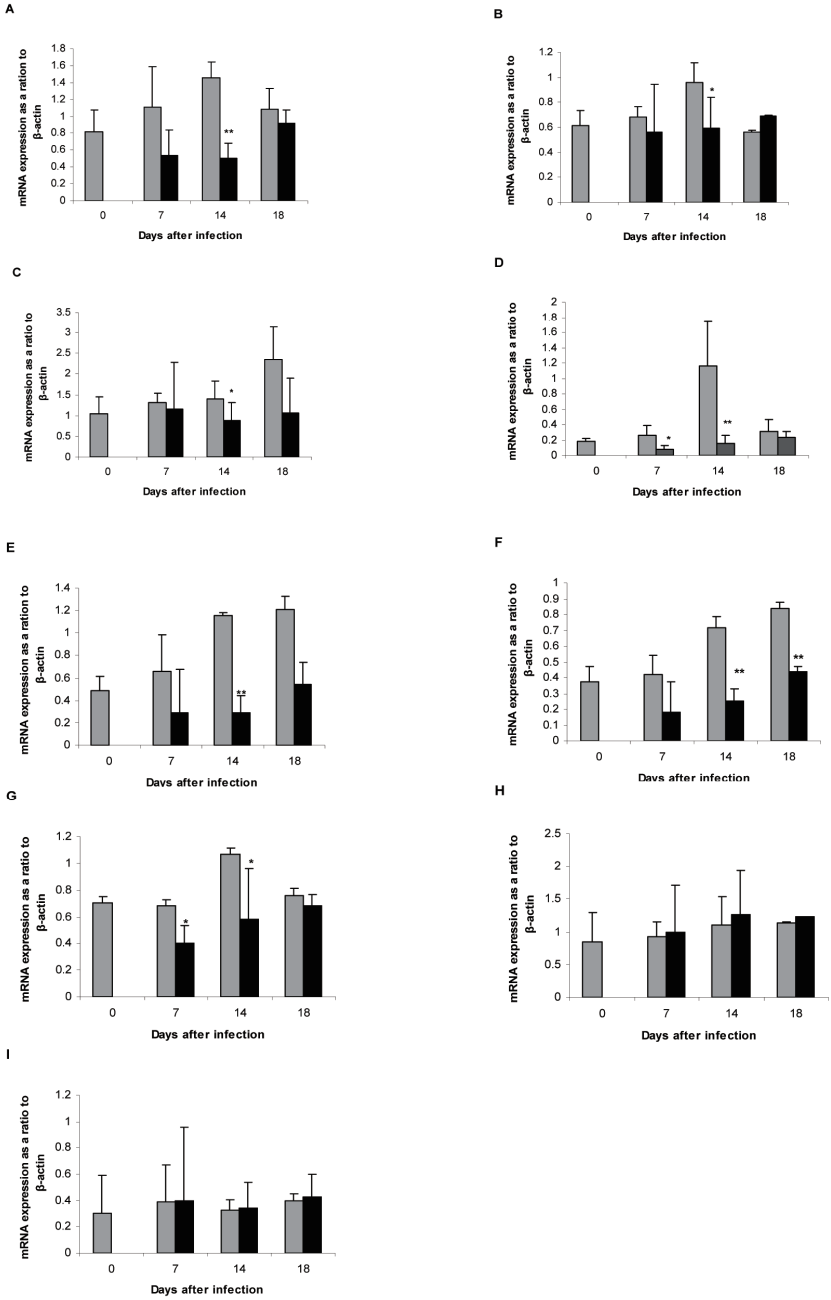

Figure 5. mRNA levels of nuclear receptors Fxr (A), Pxr (B) and Shp (C), bile acid syntesis enzyme Cyp7a1 (D), basolateral and canalicular transporters Ntcp (E),Bsep (F), Mrp2 (G), Mdr2 (H) and Oatp1 (I) in BA mice (black bars) and controls (grey bars) at seven, 14 and 18 days. Relative expression levels normalized to the expression levels of β-actin are shown. *) p< 0.05. **) p< 0.01.

Hepatobiliary transport

mRNA expression of nuclear receptors and transporters

Expression of nuclear receptors and transporters is depicted in Figure 5. Expression of *Fxr* and *Shp* decreased, reaching significance at 14 days. After 18 days, expression of both nuclear receptors increased again reaching levels not significantly different from control mice. Down-regulation of *Bsep* paralleled the lower expression of *Fxr*, also reaching significance at 14 days. A similar pattern was observed in the expression of *Pxr*, although differences compared with control mice were less pronounced.

Expression of *Ntcp* was lower in sick mice compared with control mice, reaching significance at 14 days. In contrast to *Ntcp*, the expression levels of *Oatp1* did not change during the experimental period. Significant down regulation of *Mrp2* was observed at seven days and 14 days. In comparison, the expression of *Mdr2* remained relatively unchanged. The expression of *Cyp7a1* of sick mice was significantly down-regulated at seven days and 14 days.

Protein expression of canalicular and basolateral transporters

Protein levels in total liver membrane isolates was studied at 14 days after inoculation, as most significant changes on mRNA levels were observed at that time point. The protein level of *Ntcp* was significantly down regulated, while the protein level of *Bsep* was up regulated compared to the control group **(**Fig *6*)

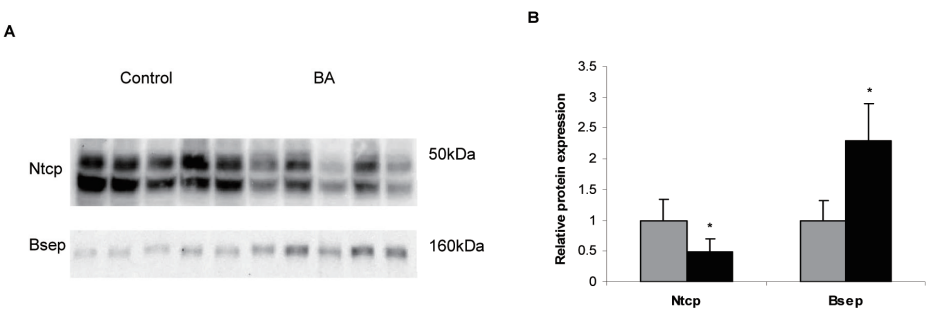

Figure 6. Western blots of Ntcp and Bsep in BA mice (black bars) and control mice (grey bars) at 14 days. (A) Western blot analysis of 50-kDa Ntcp and 160-kDa Bsep from liver extracts in control and sick group. (B) The levels of protein expression were normalized to that of the control group, and the level of control group was set as 1.0. *) p< 0.05.

Discussion

We hypothesized that inflammation might lead to a down-regulation of hepatobiliary transport in the RRV induced BA model. We demonstrated that inflammation-related down-regulation of basolateral and canalicular hepatobiliary transporters led to intrahepatic cholestasis, which might contribute to the progressive liver damage observed in this model.

One week after inoculation there was a marked inflammatory response. This coincided with down-regulation of the canalicuar and basolateral transporters *Bsep*, *Mrp2* and *Ntcp*. At that time jaundice was mainly due to unconjugated bilirubin. Despite high bile acid levels in the liver, mRNA levels of *Fxr*, *Shp* and *Pxr* started to decrease. At 14 days, mRNA expression of *RRV-Nsp3, Tnfa, Ifng* decreased, while *Il6* peaked. Hepatobiliary transporter expression remained decreased. At this point the high bilirubin levels consisted mainly of conjugated bilirubin, suggestive of bile duct obstruction which could indeed be observed microscopically. At 18 days, the expression of inflammatory cytokines, hepatobiliary transporters and the nuclear receptors normalized. These changes might be attributed to the high concentration of bile acids in the absence of an inflammatory response (17).

Histology demonstrated a ductular reaction and proliferation of cholangiocytes, similar to the changes observed in human biliary atresia. There was progressive liver injury culminating in liver necrosis, which may be due to bile acid toxicity.

There are various models of experimental cholestasis in animals. One of the most widely studied is the bile duct ligation model (BDL). After BDL there is down-regulation of *Ntcp* mRNA. This is considered to prevent further bile acid uptake by hepatocytes (18). *Oatp1* and *Mrp2* are down–regulated as well (19, 20) while *Bsep* mRNA expression is maintained. This might reduce the extent of liver injury produced by bile acid retention (21). Many of these effects are induced via activation of *Fxr* due to the accumulation of bile acids.

Another model is the LPS-induced (inflammatory) cholestasis model (22-28). In this model there is a marked down-regulation of *Ntcp*, *Mrp2* but also a down-regulation of *Bsep* mRNA expression. Several inflammatory cytokines such as *Tnfa*, *Il1β*, *Il6* are considered to repress the gene expression of both basolateral and canalicular bile transporters (29). Previous studies suggested that at early stages of

inflammation, *Ntcp* down-regulation is regulated by *Tnfa* and *Il1β* and at later stage by *Il6*(30, 31). In these studies *Il6* levels peaked at a later stage when compared to *Tnfa* levels, just as observed in the present study. *Tnfa* or *Il1β* also significantly decreases *Fxr* mRNA expression and binding activity to the IR-1 response element located in both human and rodent *Bsep* promoters (32).

In the present study, we observed a correlation in time between inflammatory response and transporter expression. These results seem to coincide with those observed in LPS-induced cholestasis, with inflammation overriding possible adaptive changes due to the increase in bile acids. As transcriptional control of *Ntcp*, *Bsep* and *Mrp2* is mediated by the nuclear receptors *Fxr* and *Pxr*, the decrease in *Fxr/Pxr* activation might be another mechanism diminishing expression of these transporters (33). The pattern of bile transporter regulation in the early stages of murine biliary atresia thus differs from acute non-inflammatory cholestasis like bile duct ligation.

Only a few studies have addressed hepatobiliary transporter regulation in human biliary atresia (34). Chen et al (35) investigated hepatobiliary transport in human biliary atresia at different stages of the disease. At early stages of biliary atresia induced cholestasis in children, most basolateral and canalicular transporters and nuclear receptors were down-regulated. This is similar to the findings of the present study. This observation underlines the validity of the mouse model for studying the pathogenesis of biliary atresia. This overall response could act to decrease intracellular bile acid levels as well as to reduce the biliary pressure. However, in humans *Fxr-Bsep* level tended to return to normal level at later stages, and *Pxr* level remained decreased, which differs slightly from the results of our study. This difference might be due to relatively short survival period of the mice in the present study.

We observed a difference between mRNA levels and protein levels of *Bsep*, in which the transcript level was down-regulated whereas the translational level was up-regulated. The level of *Bsep* down-regulation might be underestimated by the Western blot because the crude membrane techniques used in the present study includes the endosomal compartment in which still a high level of *Bsep* might be present (36, 37). Furthermore, retrieved transporter molecules may still be contained in this preparation (36). Western blotting using only basolateral or canalicular membrane preparations would yield more detailed information. Due to

the small amount of liver tissue available from pups, those techniques are virtually impossible to apply in this particular type of study.

Although regulatory effects of inflammation on hepatobiliary transport have been proven both in vivo and in vitro (38-40), the exact nature of this relation in vivo is yet not known as many inflammatory mediators might be involved. In the present study, we have shown a temporal relation between inflammation and transporter down-regulation. In the absence of knowledge about the exact mechanism by which inflammation decreases hepatobiliary transport a more direct causal relation can not be established. Experiments to that extent, in our opinion, are not possible within the present model. For instance, anti-inflammatory drugs such as steroids have profound effects on hepatobiliary transport itself, probably via the glucocorticoid receptor (41). Blocking the inflammatory response using for instance Ifng knockout mice is also impossible, as these animals rarely develop BA (42). Therefore we consider the present data the best possible answer to confirm the hypothesis that inflammation mediates down-regulation of hepatobiliary transport during the early phases of BA development.

Conclusion

Intrahepatic cholestasis due to down-regulation of basolateral and canalicular hepatobiliary transporters is an early event in the development of biliary atresia. This down-regulation is probably due to the inflammatory process, which apparently overrides the regulatory effects of the concomitant increase in bile acids in the liver. The net result is intrahepatic cholestasis and liver damage, which occurs already before extrahepatic biliary obstruction. The results of the present study are in line with preliminary studies in human, offering further proof of the validity of the mouse model and opening research approaches to mechanistical studies and possible therapeutic interventions.

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Table1. Sequences of primers, probes and PCR product sizes for amplified products.

Gene (Gene ID)	Primers	Probes	Annealing temperature	Product size
β-actin (Actb) (11461)	(F): 5'-AGCCATGTACGTAGCCATCCA (R): 5'-TCTCCGGAGTCCATCACAATG	TGTCCCTGTATGCCTCTGGTCGTACCAC	59ºC	81bp
Tnf-a (Tnf) (21926)	(F): 5'-GTACCCACTCGTAGCAAAC (R): 5'-AGTTGGTTGTCTTTGAGATCCATG	CGCTGGCTCAGCCACTCCAGC	60ºC	76bp
Ifn-g (Ifng) (15978)	(F): 5'-CCAAGCGGCTGACTGAACTC (R): 5'-TCACTGCAGCTCTGAATGTTTCTTAT	ACAGGCTGTCCCTGAAAGAAAGCAGTGTC	60ºC	82bp
Il-6 (IL6) (16193)	(F): 5'-CCTTCCTACCCCAATTTCCAAT (R): 5'-GTCCTTAGCCACTCCTTCTGTGA	AGTTGCCTTCTTGGGACTGATGCTGG	60ºC	68bp
RRV-Nsp3 (RRVgp1) (1489706)	(F): 5'-TGATTGGAAATCAAGATATGAACAGTTAG (R): 5'-TTTCTAGCTTTAAGAACCCAATGATTATAC	TGAAAGAAAGCAGTGTCCAGGCTGT	60ºC	97bp
Ntcp (Slc10a1) (20493)	(F): 5'-ATGACCACCTGCTCCAGCTT (R): 5'-GCCTTTGTAGGGCACCTTGT	CCTTGGGCATGATGCCTCTCCTC	60ºC	111bp
Oatp1 (Slco1a1) (28248)	(F): 5'-CAGTCTTACGAGTGTGCTCCAGAT (R): 5'-ATGAGGAATACTGCCTCTGAAGTG	TGGATTTGCCAGTACATTTACCTTCTTGCC C	60ºC	111bp
Bsep (Abcb11) (27423)	(F): 5'-CTGCCAAGGATGCTAATGCA (R): 5'-CGATGGCTACCCTTTGCTTCT	TGCCACAGCAAT TTGACACCCTAGTTGG	60ºC	117bp

Mdr2 (Abcb4) (18670)	(F): 5'-GCAGCGAGAAACGGAACAG (R): 5'-GGTTGCTGATGCTGCCTAGTT	AAAGTCGCCGTCTAGGCGCCGT	60ºC	64bp
Mrp2 (Abcc2) (12780)	(F): 5'-GGATGGTGACTGTGGGCTGAT (R): 5'- GGCTGTTCTCCCTTCTCATGG	AGCTGCATCGTCAGGAAT TTC CTC CAC A	60ºC	83bp
Fxr (Nr1h4) (20186)	(F): 5'- CGCTGAGATGCTGATGTCTTG (R): 5'- CCATCACTGCACATCCCAGAT	ATG ATC ACA AGT TCACCCCGCTCCTCT	60ºC	82bp
Pxr (Nr1i2) (18171)	(F): 5'- ATAGGGTTACAGCACGAACTCAGA (R): 5'- GCCATTTCAGCTTGGTCTTCTT	CGAGCTGCTTCTGTGTTCCAGACGC	60ºC	68bp
Shp (Nr0b2) (23957)	(F): 5'- AAG GGC ACG ATC CTC TTC AA (R): 5'- CTG TTG CAG GTG TGC GAT GT	ATG TGC CAG GCC TCC GTG CC	60ºC	99bp
Cyp7a1 (13122)	(F): 5'- CAG GGA GAT GCT CTG TGT TCA (R): 5'- AGG CAT ACA TCC CTT CCG TGA	TGCAAAACCTCCAATCTGTCATGAGACCTC C	60ºC	121bp

PART II

Clinical aspects of human biliary atresia and other pediatric liver diseases

CHAPTER 5

Partial external biliary diversion in children with progressive familial intrahepatic cholestasis and Alagille's disease

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Abstract

Partial external biliary diversion (PEBD) is a promising treatment for children with progressive familial intrahepatic cholestasis (PFIC) and Alagille's disease. Little is known about long term outcomes.A retrospective chart review of all patients undergoing PEBD in the University Medical Centre of Groningen (UMCG).

Between 2000 and 2005 PEBD was performed in 14 children with severe pruritus. (PFIC 11, mean age 5.3 ± 4.4 years; Alagille's 3, mean age 7.4 ± 4.2 years). Stature was < -2 standard deviation score (SDS) in 50%. Median pre-operative serum bile salt concentration was 318µmol/L (range 23-527). 29% had severe liver fibrosis, 71% mild/moderate fibrosis. Median follow-up was 3.1 years (range 2.0 to 5.7). One patient (7%) underwent a liver transplantation at 3.2 years post PEBD. Two years postoperatively 50% were without pruritus, 21% had mild pruritus. In 29%, pruritus had not diminished, three of them had severe fibrosis pre-operatively. In patients with mild/moderate fibrosis, PEBD decreased serum bile salts (105µmol/L (range 8-269) two years postoperatively). Bile salts did not decrease in patients with severe fibrosis. Two years after PEBD, 27% had a stature below -2 SDS. At median follow-up of 3.1 years after PEBD, pruritus has been relieved in 75%. Bile salts level and growth are improved in most of patients. Longer follow-up is needed to determine whether PEBD can postpone or avoid the demand for liver transplantation.

Introduction

Progressive Familial Intrahepatic Cholestasis (PFIC) represents a group of autosomal recessive disorders, characterized by progressive hepatocellular cholestasis. Subsequent retention and accumulation of hydrophobic bile salts within the hepatocyte lead to progressive liver damage. Alagille's disease is a syndrome consisting of (among others) persistent cholestasis, cardiac anomalies, butterfly vertebrae and dysmorphic facies. Becoming manifest within the first year of life, these disorders often progress to cirrhosis and subsequent hepatic failure before the end of the second decade. Children present with jaundice, diarrhea and cholestasis. The main complaint is often the intractable pruritus, sometimes leading to severe automutilation. Growth failure and nutritional deficiencies secondary to long chain fat and fat soluble vitamin malabsorption may also occur.

Mutations in hepatocellular transport system genes involved in bile formation seem to be the underlying defect in all types of PFIC, leading to impaired hepatobiliary transport. Liver biopsies in PFIC show hepatocanalicular cholestasis, fibrosis and finally cirrhosis, while in Alagille's disease there is cholestasis and a paucity of bile ducts.

Medical treatment of both children with PFIC and children with Alagille's disease is aimed at relieve of pruritus and slowing the progression of disease. However, medical treatment often fails, and severe pruritus and/or end-stage liver disease due to PFIC or Alagille's disease has been among the most common indications for orthotopic liver transplantation in children. An alternative surgical procedure using partial external biliary diversion (PEBD) has gained popularity over the last years (1). This procedure interrupts the entero-hepatic circulation of bile salts by partially diverting bile from the gallbladder through a loop of jejunum connecting the gallbladder with the abdominal skin. The influx of bile salts into the gut and their subsequent reuptake are thus diminished, decreasing the pool of bile salts.

Earlier reports describe alleviation of pruritus, improved liver chemistry and liver function tests, improved serum lipid levels and reversed growth failure after PEBD. However, mid or long-term follow-up is often not available. In this report we describe the results of PEBD in children with PFIC or Alagille's disease with a minimal follow-up of two years.

Methods

Patients

A retrospective chart review was performed of all patients who had undergone PEBD for PFIC or Alagille's disease at the University Medical Centre Groningen between 2002 and 2005. The diagnosis of PFIC and Alagille's disease was established primarily on clinical grounds, laboratory parameters and liver biopsy.

Surgery and follow-up

Surgery was performed by or under close supervision of experienced paediatric and/or hepatobiliary surgeons. In brief, via a sub costal incision a 10- to 15 cm jejunal conduit was constructed from the dome of the gallbladder to the abdominal wall in the right upper quadrant. The proximal portion of jejunal conduit was anastomosed end to end or end to side (depending on the preference of the surgeon) to the gallbladder dome. The posterior side of the anastomosis was performed using interrupted sutures, and the anterior side was performed using a running suture. The distal end of the jejunal conduit was brought out to the skin as permanent ostomy (figures 1 and 2). The continuity of the remaining jejunum was restored by end to end anastomosis. Liver biopsies were performed in all cases during surgery. Fibrosis was scored by an experienced liver pathologist as mild (portal fibrosis without septa), moderate (portal fibrosis with septa) or severe fibrosis/cirrhosis (septa without cirrhosis/nodular transformation)

During follow-up patients were seen at regular intervals at the paediatric gastroenterology outpatient clinic. No liver biopsies were obtained during follow-up. Liver function was regularly tested. Bile composition was not assessed on a routine basis.

Outcome Measures

Main outcome measure was transplantation-free survival, indexed by the time of entering the waiting list for liver transplantation. Secondary outcome measures were the severity of pruritus, growth (standard deviation scores for height preoperatively and two years postoperatively were calculated using growth charts

for Dutch children, with a deviation of > -2 standard deviation of the mean for age considered as pathological), biochemical parameters (including total bile salts and liver function tests), and quality of life (estimated by the severity of pruritus, sleep of patients and parents and the resumption of normal daily (school) activities. The use of medication and vitamin suppletion was retrieved from the charts, as was the presence of extrahepatic symptoms such as diarrhoea, pancreatitis en hearing problems. Surgical morbidity and mortality were assessed (length of hospital stay, number and kind of complications, number of re-operations).

Statistical analysis

Statistical analysis was performed using the SPSS 14.0 statistical software package. For continuous variables, Student's t-test or Mann-Whitney U test were applied, for categorical variables Chi-square or Fisher Exact tests were used.

Results

Patients

Eleven children with PFIC underwent PEBD: 2 boys and 9 girls at a mean age of 5.3 ± 4.4 years at the time of surgery. Eight of the 11 PFIC patients had a genetically proven PFIC II (mutations in BSEP gene). Three patients who were diagnosed with Alagille's disease underwent PEBD: 1 boy and 2 girls, mean age 7.4 ± 4.2 years at the time of surgery. There were three families in which multiple members were affected: two patients were twins (Alagille's), two were sisters (PFIC) and two other patients (PFIC) were children from consanguineous parents (who were first cousins). All patients suffered from intractable pruritus with significant excoriations despite optimal medical treatment. All used vitamin suppletion, eight used rifampicin and ten used ursodeoxycholic acid. In one patient an ileo-colonic bypass two years before the biliary diversion had not been successful. No patient suffered from diarrhoea or pancreatic insufficiency.

Pre-operative laboratory results are depicted in table I. Characteristically, there was a marked increase in total bile salts, with normal values of serum gamma-glutamyltransferase in patients with PFIC as compared to patients with Alagille's disease. Serum aminotransferases were only slightly elevated. At the time of

surgery all patients had histologies compatible with PFIC or Alagille's disease, showing lobular cholestasis without ductular proliferation, and various degrees of portal and lobular fibrosis or cholestasis and paucity of bile ducts, respectively. Of the PFIC patients two had mild fibrosis of the liver in their liver biopsies; seven had moderate fibrosis and two had severe fibrosis. Two of the Alagille patients had severe fibrosis.

Surgery

Initial surgery was uneventful in all patients. Two patients underwent a relaparotomy for early postoperative complications (postoperative bleeding at day three; wound dehiscence at day two). Median hospital stay was eight days (range 6 to 28), without mortality. In three patients a parastomal hernia or stoma prolapse developed, which had to be corrected surgically. One patient underwent a dilatation of the stoma.

Follow-up

Median follow-up was 3.1 years (range 2.0 to 5.7 years). Overall one patient (7%) underwent a liver transplantation at 3.2 years post PEBD, in two children liver transplantation is considered at present due to intractable pruritus. These children include the two patients with severe fibrosis preoperatively. In the group with PFIC, five children (45%) did not suffer from pruritus two years post-PEBD. Three (27%) had mild pruritus, three (27%) still suffered from severe pruritus with significant scratch marks. In the children with Alagille's syndrome, one patient still suffered from intractable pruritus, the others were free of pruritus.

Mean standard deviation score for growth (SDS) was -2.4 ± 0.3 preoperatively and -1.8 ± 0.3 at two years after PEBD (PFIC; p=0.05). Corresponding values were -1.3 ± 0.3 and -1.4 ± 0.6 for Alagille (NS). Preoperatively, 63% of patients had an SDS < - 2.0 in PFIC, two years postoperatively this was 27% (p=0.21). There were no patients with SDS scores < -2.0 in the Alagille group, neither preoperatively nor postoperatively.

The sleep of patients and parents was improved, and all patients resumed their school activities, thus contributing to an improved quality of life. The biliary stoma was tolerated well and stoma care was successful in all cases. The median bile

production of the biliary stoma was 200ml/day (range 40 ml – 450 ml). There were no hospital readmissions due to cholangitis.

In the PFIC group, ten patients used vitamin suppletion (vitamins, A, D, E and K). Six patients still used rifampicine, and six patients used ursodeoxycholic acid. In the Alagille group all patients used vitamin supplements, the patient with severe pruritus used rifampicin.

Laboratory results are depicted in table 1. In PFIC, all liver function tests tended towards improvement, although only LDH and total serum bile salt levels showed a statistically significant decrease after two years of follow-up. (figure 3) Decrease in total bile salt levels were most pronounced in patients with mild fibrosis (from 247 umol/L preoperatively to 19 umol/L at two years follow-up), and absent in patients with severe fibrosis (from 368 umol/L to 350 umol/L). The synthetic function of the liver remained intact, indicated by adequate albumin levels and normal coagulation. In Alagille's disease there was no significant change in laboratory values over the years.

Table 1. Laboratory findings in 11 patients with PFIC and three patients with Alagille's disease, direct pre-operatively, one year postoperatively and two years postoperatively.

	Normal values	Preop median (range)	1 year postop median (range)	2 years postop median (range)
ALAT (μ/L)	0–30			
PFIC		75 (26–611)	45 (23–222)	41 (18–250)
Alagille		77 (70–309)	356 (233–480)	199 (162–367)
ASAT (μ/L)	0–40			
PFIC		121 (34–593)	67 (28–367)	65 (925–271)
Alagille		96 (86–327)	310 (159–461)	169 (161–250)
γ-GT (μ/L)	0–65			
PFIC		20 (15–37)	14 (10–102)	15 (9–41)
Alagille		431 (401–519)*	384 (289–479)*	411 (248–539)*
ALP (μ/L)	13–120			
PFIC		383 (192–661)	355 (167–909)	370 (194–1122)
Alagille		844 (737–1242)	1298 (1156–1441)	918 (510–1514)
Bilirubin (μmol/L)	7–17			
PFIC		33 (16–142)	10 (2–80)	12 (2–92)
Alagille		77 (21–306)*	160 (13–307)*	161 (14–224)*
LDH (μ/L)	114–235			
PFIC		374 (232–635)	333 (220–432)	308 (200–439)†
Alagille		330 (287–379)	374 (280–469)	259 (241–408)
PT (s)	11–16			
PFIC		14.0 (11.5–18.6)	13.9 (11.7–14.3)	12.3 (11.1–13.1)
Alagille		12.7 (12.6–13.1)	13.7 (12.8–14.6)	13.1 (11.0–14.1)
Albumin (g/L)	34–47			
PFIC		44 (38–51)	44 (42–50)	46 (42–80)
Alagille		44 (39–45)	44 (43–45)	44 (34–46)
Total bile salts (μmol/L)	1.1–8.9			
PFIC		346 (23–527)	189 (12–939)	105 (8–269)†
Alagille		274 (242–318)	149 (49–250)	66 (39–263)

¶: significance difference between PFIC and Alagille;

*** significant difference between preoperatively and 2 years postoperatively**

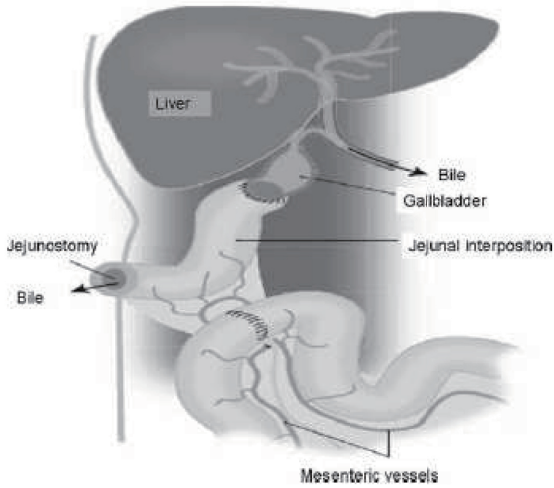

Figure 1. Partial external biliary diversion (PEBD) consisting of an isoperistaltic jejunal conduit between the dome of the gallbladder and the abdominal skin.

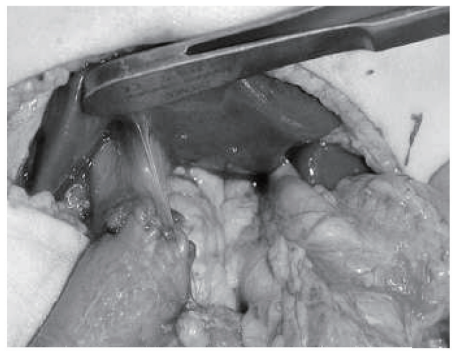

Figure 2. Cholecystojejunocutaneostomy in a patient with PFIC.

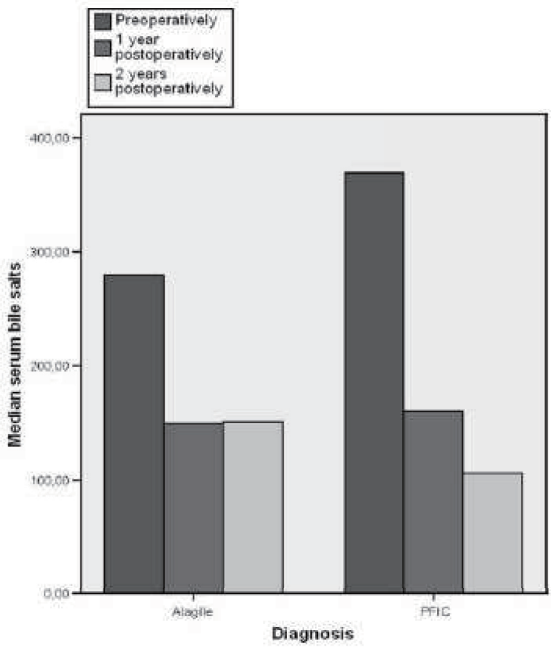

Figure 3. Median serum total bile salts pre-operatively, after one year of follow-up and after two years of follow-up in PFIC and Alagille's disease. *: P<0.05 compared with preoperative values

Discussion

We evaluated the results of PEBD for PFIC or Alagille's disease after at least two years follow-up. Pruritus decreased significantly or disappeared altogether in almost three quarter of the patients regardless of the underlying disease (PFIC or Alagille's disease). Although a formal assessment of pruritus using a pruritus score has not been performed due to the retrospective nature of this study, the results are in concordance with the literature, in which up to 85% of patients has a complete resolution of pruritus (1-5). However, in the present series approximately half of the patients still used rifampicin or ursodeoxcholic acid after two years of follow-up, indicating that pruritus had not been abolished completely by the operation. Three children (27%) with PFIC and one child with Alagille (33%), still suffered from intractable pruritus. One of them underwent a liver transplantation at 3.2 yrs post PEBD, while the others are considered for transplantation.

In the present series growth also seemed to improve, with stature below –2 standard deviation in one third of the patients after two years of follow-up. Furthermore, sleeping and daily activities were improved in most of the patients and their parents.

PEBD is a safe procedure without mortality, although in the present series minor reinterventions were necessary in 50% of patients. In the present series stoma prolapse was the main surgical complication; three patients had to undergo surgical correction of the prolapse. The occurrence of cholangitis should be minimized by the placement of a pro-peristaltic conduit between the biliary tree and the skin. In this series there were no bouts of cholangitis leading to hospital readmission, which is in accordance with the low incidence of cholangitis mentioned in the literature. PEBD might even be performed laparoscopically, although experience is limited (6). Ostomy care was successful, and so far there have not been any problem with acceptance of the ostomy. However, most patients in our series have not reached puberty yet.

A series of surgical alternatives are considered to decrease bile salts. Direct anastomosis of the gall bladder to the skin is supposed to be the most direct method to PEBD, while this results high risk of cholangitis, and maintaining an adequately watertight stoma is also difficult (7). The diversion of bile to the urinary

system has been also proposed; however, it is not favored due to urine reflux into the biliary system and a lack of knowledge about long-term effects of chronic biliary irritation on the urinary epithelium (8). Therefore, those alternatives do not seem to be satisfactory procedures.

Another surgical technique making use of the partial diversion of bile flow yet avoiding an external biliary fistula is the ileocolonic bypass. During this procedure approximately 15% of the terminal ileum is excluded, thus decreasing the reuptake of bile components. Although first results were similar to those after PEBD, long term results demonstrated recurrence of symptoms in over 50% of patients, which seems inferior to the results of PEBD (9). In our series one patient underwent an ileocolonic bypass which had to be converted two years postoperatively to a PEBD. Recently Bustorff-Silva et al described two patients in which partial biliary diversion was achieved by performing a cholecystojejunocolonic anastomosis, combining the advantages of partially diverting bile flow and avoiding an external biliary fistula (10). However, longer follow-up is necessary to evaluate the results and complications of this procedure.

The working mechanisms of PEBD are not completely elucidated. Interruption of the enterohepatic circulation of bile salts results in a decrease of the bile acid pool, lessening the burden of the deficient transport mechanism. In our series, there was a significant decrease in the level of total bile salts (from 346 umol/L preoperatively to 105 umol/L two years postoperatively), which has also been described by others (11,12). Hepatic morphology might improve significantly after PEBD for PFIC, showing an almost complete resolution of cholestasis, portal fibrosis and inflammation (12). Biliary bile acid composition might also improve, as evidenced by an increase in the proportion of chenodeoxycholic acid. In the present series liver biopsies nor were not performed during follow-up, so histological improvement could not be assessed. The synthetic function of the liver remained intact as evidenced by normal albumin levels and a normal coagulation. All liver function tests tended towards improvement, although only LDH and total serum bile salt levels showed a statistically significant decrease after two years of follow-up. This lack of statistical significance may very well be due to the low number of patients.

In our series, the results in patients with severe fibrosis pre-operatively were less favourable compared with patients with mild or moderate fibrosis. All patients with severe fibrosis still suffered from intractable pruritus at two years, accompanied by

no effects of PEBD on serum bile salt levels. This observation has also been described by others, suggesting that PEBD may be an effective treatment for PFIC and Alagille only in the absence of severe fibrosis or cirrhosis (13). Severe fibrosis in pre-operative biopsies might therefore be a contra-indication for PEBD. This finding also suggests that PEBD is more effective in earlier stage of disease and might be advocated earlier.

With the increasing success rates of liver transplantation in children, some centres advocate liver transplantation as a first option for children with PFIC, even in children without severe fibrosis or cirrhosis (14). Possible benefits of primary liver transplantation are the higher chances of receiving a liver graft in young patients and the avoidance of an ostomy (14). Also, the development of hepatoblastoma has been described in children with PFIC (15,16). However, liver transplantation carries significant morbidity and mortality, and there is the need for life-long immunosuppression.

By relief of pruritus, improving growth and resolution of biochemical abnormalities PEBD might postpone or preclude liver transplantation in patients with PFIC. Postponing liver transplantation might enable patients to optimize nutritional and general health status before transplantation. The possible reversal of histological and ultrastructural lesions might be an indication that liver transplantation might not be indicated in this group of patients. In the present study, liver transplantation was performed on one patient (7%) at 3.2 years after PEBD. However, in our daily practice, postoperative biopsy to evaluate the change of liver fibrosis compared to preoperative condition was not performed routinely, so these data on histology were not available in the present study. Longer follow-up with formal assessment of quality of life, biochemical and histological parameters after PEBD are needed to assess the ultimate results of this procedure. Until new treatment modalities such as gene therapy might become available, PEBD remains the first line of treatment in patients with PFIC (17). A strict follow-up protocol is needed to early identify patients who are candidates for liver transplantation.

Conclusion

The present series shows that PEBD can be a successful treatment option for PFIC or Alagille's disease in the absence of severe fibrosis or cirrhosis. It is a relatively safe procedure, with acceptable morbidity and no mortality. After a follow-up of at least two years, pruritus has been relieved in three quarters of patients, growth has accelerated and biochemical parameters have improved in most patients. However, in 28% of patients PEBD does not diminish pruritus, leaving liver transplantation as the only option. Longer follow-up is needed to decide whether PEBD can only function as a bridge to transplantation or might abolish the need for transplantation altogether.

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CHAPTER 6

Steroids after Kasai portoenterostomy for biliary atresia: a systematic review and meta-analysis

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Submitted

Abstract

There has been a controversy about the possible role of treatment with steroids after Kasai procedure. A systematic review was performed of the English literature published between 1995 and January 2008, with emphasis on the differences in outcome between patients with or without steroid treatment in terms of clearance of jaundice at six months; of 1-year, 3-year and 5-year survival, and of incidence of cholangitis. Twenty studies were included: one randomized trial, one prospective single-centre study, seven retrospective comparative studies, and eleven retrospective non-comparative studies. Overall, clinical outcome parameters were analyzed from 1175 patients with and 645 patients without steroids treatment. The percentage of patients with adequate clearance of jaundice was significantly higher in the steroids group (60% vs. 40%; odds ratio 2.16, 95% CI 1.08 – 4.35). Transplantation free survival seemed also to be increased in the group of patients receiving steroids (1*yr*, 65% vs. 51%; 3*yr*, 72% vs. 30%; and 5 *yr*, 58% vs. 36%, respectively). The three-year survival difference was statistically significant (p=0.03, odds ratio=6.0, 95% CI 1.40-26.50). Even though these results need to be confirmed further by a large-scale clinical trail, the present meta-analysis is still the best evidence available.

Introduction

Biliary atresia (BA) is a progressive inflammatory obstruction and fibro-obliteration of the extrahepatic biliary tree (1). Bile flow can be re-established in up to 60-80% patients by performing a Kasai portoenterostomy in which the fibrotic hilar plate is resected and a Roux-Y portoenterostomy is performed. Despite a technically successful Kasai operation, survival with native liver is only around 20% to 30% at 10-year follow-up, even in experienced centres (1). Therefore, a more effective treatment is in demand to optimize the prognosis of patients with biliary atresia.

One of the most important strategies to improve outcome is early referral (which might be improved using a stool colour card) (2). Adjuvant medical therapy such as post-operative administration of antibiotics to decrease the incidence of cholangitis might also improve outcomes (3). Another option is post-operative treatment with steroids. The main theoretical reasons for postoperative treatment with steroids are its choleretic (4) and anti-inflammatory effects, which are suggested to reduce bacterial colonization of the biliary tree by increasing bile flow and to reduce periductal inflammation and edema (5). This might improve clinical outcomes by decreasing the incidence of cholangitis and decreasing the incidence of recurrent fibrosis at the portoenterostomy site. On the other hand, the detrimental aspects of steroids (such as wound dehiscence, gastrointestinal hemorrhage and infection) should be taken into account. Although there are small prospective studies on the efficacy of steroids treatment, which suggest that steroids are not benificial, the numbers in these studies are limited, and study design is often debatable.

The purpose of the present study was to perform a systematic review and subsequent meta-analysis of the outcomes of biliary atresia patients who underwent a Kasai portoenteromy, comparing short and long term outcomes between patients who received steroids treatment postoperatively and patients who did not receive steroids treatment.

Patients and methods

A literature search (Medline) was performed and all studies published in the English literature between 1995 and 2008 describing the postoperative course after portoenterostomy were identified, using the following Medical Subject Headings (MESH): biliary atresia, surgery and steroids. All titles and abstracts were scanned, and related citations were reviewed. A manual search of the bibliographies of relevant papers was also carried out to identify publications for possible inclusion. The search was performed by two independent investigators (HY and JBFH).

Both comparative and non-comparative studies were included. When there were more articles reporting on the same patient-material, only the last paper was included. When comparative and non-comparative studies reported the cases from the same medical centre at the similar period, only the comparative study was included to avoid the overlap between the two studies. Care was taken to avoid inclusion of (covert) double publications. Studies reported by the same institution or the same author were scrutinized by two of the present researchers (HY and JBFH). Articles in which administration of steroids could not be identified clearly were excluded. Also the papers which did not provide the main parameters identified for the present systematic review were excluded. Patients who received postoperative steroids treatment were regarded as one entity without taking the different treatment regimes into account.

The analysis was performed with two different primary outcomes: clearance of jaundice at six months as a parameter for short term outcome (defined as total bilirubin level less than 20 μmol/L at six month after surgery) and transplantation free survival (with a maximum of five-years of follow-up) as a parameter for long term outcome. The incidence of cholangitis was also assessed. In this analysis we combined all data on cholangitis into one item disregarding the different definitions (such as fever accompanied by evaluated bilirubin levels and leukocytosis, or change of yellow stool to alcoholic stool, or deteriorating liver functions). Age at Kasai, a readily available confounding factor, was also assessed.

Statistical analysis

Statistical analysis was performed using the NCSS package. Studies were divided into two groups, comparative studies and non-comparative studies. For each trial, 2x2 tables were constructed for the number of patients treated with steroids and the number of patients not treated with steroids with regards to clearance of jaundice at six months, transplantation-free survival and the occurrence of cholangitis. When data concerning specific topics were not stated, the study under review was excluded for that topic only. Odds ratio was calculated with the group of patients not treated with steroids as the reference group, so that treatment benefit for patients receiving steroids are associated with an odds ratio above 1. To compare between groups, Chi-square test was used for categorical parameters, considering a p-value below 0.05 as statistically significant.

Tests for homogeneity (Effect-Equality Test) of the odds ratio were performed for the main short-term endpoint 'clearance of jaundice'. Based on published survival rates in the different studies, we calculated survival at one, three and five years for the combined studies. Since there was no uniform definition of "cholangitis" and data about the time of follow-up were not available, we decided to depict data in each comparative study separately.

Results

Twenty studies were included addressing the questions raised (Table 1). There were nine comparative studies, one randomized trial, one prospective single-centre study and seven retrospective studies. Seven retrospective non-comparative studies described results of patients who received steroids post-Kasai, while the remaining four studies are retrospective studies reporting on patients who did not receive steroids postoperatively. Test for homogeneity (Effect-Equality Test) demonstrated a slight heterogeneity of the odds ratios (p=0.033), when including both randomized trial and comparative studies for the comparison of clearance of jaundice. There was no heterogeneity, when only comparing comparative studies for this parameter (p=0.08). Age at Kasai was similar in the patients who did or did not receive steroids (61 vs. 64 days).

Table 1. Articles included according to the criteria outlined in the method section.

	Reference[number]	No. of Patients	years	Study type
Comparative studies	*Davenport et al, 2007* [6]	*73*	*2000-2005*	*Randomized*
	Petersen et al. 2008 [7]	49	2001-2004	Prospective
	Meyers et al, 2003 [8]	28	1994-2002	Retrospective
	Muraji et al, 2004 [9]	222	1997-2000	Retrospective
	Kabayashi, et al. 2005 [10]	63	1985-2003	Retrospective
	Escobar et al, 2006 [11]	43	1992-2004	Retrospective
	Shneider et al. 2006 [12]	104	1997-2000	Retrospective
	Stringer et al, 2007 [13]	60	1994-2006	Retrospective
	Vejchapipat et al. 2007 [14]	53	2001-2005	Retrospective
Overall comparative studies		695	1994-2006	
Non-comparative studies				
No steroids	Michael Oh et al, 1995 [15]	62	1978-1992	Retrospective
	Davenport et al, 1997 [16]	362	1974-1995	Retrospective
	Wildhaber et al, 2003 [17]	81	1974-2001	Retrospective
	Lykavieris et al, 2005 [18]	271	1968-1983	Retrospective
Steroids	Muraji et al, 1997 [19]	14	1990-1996	Retrospective
	Hashimoto et al, 1997 [20]	40	1988-1995	Retrospective
	Muraji et al, 1997 [21]	42	1986-1994	Retrospective
	Dillon et al, 2001 [22]	25	1991-1999	Retrospective
	Hung et al, 2005 [23]	141	1976-2000	Retrospective
	Shimadera et al, 2007 [24]	29	1988-2004	Retrospective
	Lal et al, 2007 [25]	58	2000-2006	Retrospective

Table 2. Clearance of jaundice six months after Kasai portoenterostomy in patients receiving steroids and patients not receiving steroids postoperatively.

	Number of patients		Clearance of jaundice (%)		Statistics		
	No steroids	*Steroids*	*No steroids*	*Steroids*	*Odds ratio*	*95% C.I.*	*P-value*
Randomized[6]	37	36	49%	47%	0.90	0.30-2.10	0.70
Comparative [7,8,10,11,13,14]	117	189	37%	69%	2.60	1.30-5.50	<0.01
Comparative overall	**154**	**225**	**40%**	**65%**	**2.16**	**1.08-4.35**	**<0.01**
Non-comparative [17,20,22,23,25]	68	300	49%	58%	-	-	-

Abbreviations: *C.I.* = Confidence interval

Short-term effects of steroid treatment as measured by clearance of jaundice at six months are depicted in Table 2, Figure 1 and Figure 2. In the randomized trial, there was no significant difference between the patients with and without steroids treatment (p=0.7, odds ratio=0.9). In other non-randomized comparative studies, however, patients with steroids treatment were more likely to achieve adequate clearance of jaundice (p= 0.008, odds ratio=2.6). When all the comparative studies were combined, postoperative treatment with steroids led to a significantly higher

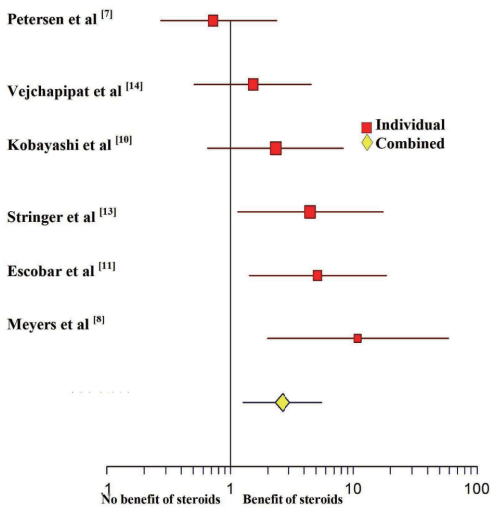

Figure1. Forest plot of the odds ratio of clearance of jaundice at six months in seven comparative studies including one randomized trial and six comparative studies

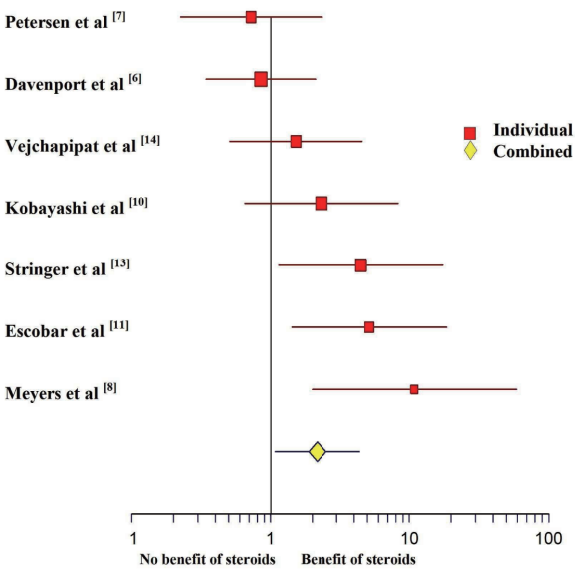

Figure2. Forest plot of the odds ratio of clearance of jaundice at six months in six non-randomized comparative studies

percentage of patients with adequate clearance of jaundice at six months (65% vs. 40% respectively, *p*=0.007, odds ratio=2.16). A similar trend was found in non-comparative studies, 59% vs. 49%. Three studies comparing intravenous long-term (1 to 3 months) or multi-course steroids treatment (based on stool monitoring) with no steroids treatment showed similar results (*p*=0.01, odds ratio=2.87) (Table 3, Figure 3).

Table 3. Clearance of jaundice six months after Kasai portoenterostomy in patients receiving long term, or multi-course intravenous steroids and patients not receiving steroids postoperatively.

	Number of patients		Clearance of jaundice (%)		Statistics		
	No steroids	*Steroids*	*No steroids*	*Steroids*	*Odds ratio*	*95% C.I.*	*P-value*
Comparative [8,10,14]	46	98	43%	71%	2.87	1.01-8.09	0.01

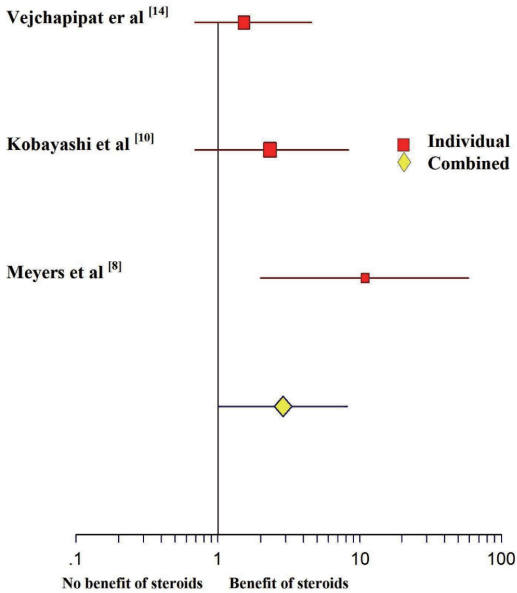

Figure3. Forest plot of the odds ratio of clearance of jaundice at six months in three comparative studies in which intravenous long-term or multi-course steroids or long-term adjuvant therapy were administered

Table 4 summarizes long-term postoperative survival with native liver. After a follow-up of one, three and five-year survival with native liver tends to be higher in patients who received steroids compared with those who did not (1*yr*, 65% *vs*. 51%; 3*yr*, 72% *vs*. 30%; and 5 *yr*, 58% *vs*. 36%, respectively). The three-year survival difference was statistically significant (p=0.03, Odds ratio=6.0). In the non-comparative studies the five-year transplantation-free survival rates were 57% and 46% in treated and non-treated patients, respectively.

Table 5 depicts the incidence of postoperative cholangitis in comparative studies. The incidence of cholangitis was lower in patients with steroids treatment in three of the five studies, but only in one of the studies the difference was statistically significant (*p*=0.02, Odds ratio=5.2).

Table 4. Postoperative transplantation-free survival in patients receiving steroids and patients not receiving steroids postoperatively at one year, three years and five years postoperatively.

	Number of patients		Survival (%)		Statistics		
	No steroids	*Steroids*	*No steroids*	*Steroids*	*Odds ratio*	*95% CI*	*P-value*
Comparative							
One year[8,12]	72	55	51%	65%	3.00	0.30-29.00	0.20
Three years[13]	10	50	30%	72%	6.00	1.40-26.50	0.03
Five years[9]	14	208	36%	58%	2.50	0.80-7.70	0.05
Non-comparative							
Five years [16,17,19, 20, 21, 22, 23, 24]	403	287	46%	57%	-	-	-

Table 5. The incidence of postoperative cholangitis in comparative studies.

Comparative studies	cholangitis		Follow up (years)	Statistics		
	No steroids	*Steroids*		*Odds ratio*	*95% CI*	*P-value*
Meyers et al[8]	43%	28%	3.8	0.50	0.10-2.70	0.70
Kabayashi et al[10]	17%	20%	-	1.20	0.20-6.50	1.00
Escobar et al[11]	59%	57%	4.0	0.90	0.30-3.10	0.90
Stringer et al[13]	80%	36%	3.3	5.20	1.20-22.60	0.02
Vejchapipat et al[14]	55%	39%	0.5	0.50	0.20-1.60	0.30

Table 6. Regimens of steroids treatment in comparative studies and randomized trials.

Reference[number]	Regimen of steroids
Davenport et al [6]	Oral,3wk: 2 mg/kg /d 2wk, followed by 1 mg/kg /d ,1wk.
Petersen et al. [7]	Intravenous and oral, 4wk: 10 mg/kg 5d followed by oral,1 mg/kg 3wk.
Meyers et al [8]	Intravenous and oral, 9-13wk: 10–2 mg/kg /d tapered 1 wk followed by oral 2mg/kg ,8–12 wk.
Muraji et al [9]	Intravenous or intravenous and oral: initial dose <3.9mg kg /d or >4.0mg/kg/d; Blast dose followed by half dose tapering, or a constant dose.
Kabayashi et al [10]	Intravenous, single course, 3d in decreasing dose: 6, 4, 2 mg/kg /d, or 10,5,2.5 mg/kg /d, or 20, 15, 10 mg/kg /d, or 20, 15, 10 mg/kg /d , and repeats when stool colour turns to pale.
Escobar et al [11]	Intravenous, 2-6wk: 2-20 mg/kg /d, with tapering for 2-6wk.
Shneider et al [12]	Single course, dose and method are not mentioned in detail.
Stringer et al [13]	Oral, 5d: dexamethasone 0.3mg/kg bid 5d, 0.3mg/kg bid 5d, 0.1mg/kg bid 5d.
Vejchapipat et al [14]	Intravenous,1-3m,alternative days: 2mg/kg/d.
BARC*	Intravenous and oral, 13wk: 1-3d Intravenous, 4mg/kg/day, 4-7d: Oral, 4mg/kg/day, with tapering to 0.1 mg/kg/day, every other day at 13wk.

***Biliary Atresia Research Consortium (BARC)**

Discussion

This meta-analysis demonstrates a significantly higher percentage of an adequate clearance of jaundice at six month in patients receiving steroids as compared with patients not receiving steroids postoperatively. Also, transplantation-free survival is increased in patients receiving steroids. However, there are only limited data available at the different time points, so these latter results have to be interpreted cautiously. A similar interpretation seems warranted for the occurrence of cholangitis, which seems to be decreased after administration of steroids post-Kasai, but definitions vary and data on follow-up time are often not available.

Only one of the studies included in this meta-analysis is a prospective placebo-controlled randomized trial, with 73 patients enrolled. This study failed to show a benefit of postoperative steroids. A similar result was obtained in a recent study by Petersen et al (7), who reported the outcomes of a prospective single centre and open-labelled pilot study in 49 patients. These outcomes are in contrast with the combined data of the comparative studies presented in the meta-analysis. One of the factors which may affect outcome is the treatment regime used.

The most promising results have been achieved by intravenous administration of steroids at higher dose. For instance, Kobayashi et al (10) demonstrated no difference between low dose steroids and no steroids post-Kasai with regards to clearance of jaundice. However, clearance of jaundice was significantly higher in patients receiving high dosage steroids. Similar results were obtained by another Japanese study (9). It cannot be excluded that the lack of beneficial effect of steroids in both the randomized trial and the single centre study by Petersen is dose or term related: the dosage or the term of administering steroids were relatively low (Table6).

Another explanation of the difference in results might be the origin of the studies, since it has been claimed that BA in Japanese population is a rather different disease entity, with a higher incidence and relatively better prognosis after Kasai compared with the data from other countries (26). However, the studies included in this review demonstrating improved results after the administration of steroids are also from European countries, not only from Japan.

Outcomes may also be affected by other therapies besides steroids. In 2001, Dillon et al (18) reported improved clinical outcomes within five years after surgery (including clearance of jaundice and survival with native liver) by adopting the combination of steroids and antibiotics. There is scanty data on the postoperative use of antibiotics after Kasai portoenterostomy, and could not be assessed in the present study, although a publication bias my not be ruled out.

It should be realized that limitations of the present review could have affected the outcomes. There are relatively few papers addressing this issue, and only one randomized trial is included. Probably, the low incidence of biliary atresia (1:16,000-20,000) contributes to this. The meta-analysis is thus performed mainly on comparative non-randomized studies. This weakens the conclusions. Also, most studies included in this meta–analysis were not specifically designed to evaluate the outcome of steroids versus no steroids. It should however be taken in account that biliary atresia is a rare disease treated in paediatric centres and that the reported studies most likely do not have a selection bias in reporting the cases.

Another limitation is the total number of patients in comparative studies, only 695. Also, dosage regimens vary between the studies, and outcomes are not reported uniformly. Other confounding factors are e.g. the timing of surgery, the extent of dissection and other surgical variables, the experience of the team caring for the patient, and the use of antibiotics postoperatively. These data are – with the exception of age at Kasai, which is not different between both groups – not available for comparison, and therefore may be a source of bias.

Furthermore, when the overall effect was calculated based on randomized trial and comparative studies, the slight heterogeneity might weaken the overall results. Therefore, we did further calculations on the comparative studies only, where heterogeneity did not exist. A similar resulted was achieved.

The only option to overcome the drawbacks mentioned above is to perform a randomized, placebo-controlled and double-blinded study. Currently, the Biliary Atresia Research Consortium (BARC) is conducting a clinical trial to evaluate whether long-term, high dose treatment with steroids improves the outcomes of Kasai in patients with BA. This clinical trial is expected to take seven years (2005 to 2012), and ten major medical centres in US are involved (Table 6). Until such a study is completed, the present meta-analysis suggesting a benefit for steroids is the best evidence available.

Conclusion

There is a discrepancy between the results of the only (small) randomized trial which does not show a benefit of steroids and the combined results of the other studies which do demonstrate such a benefit of steroids treatment after Kasai. Based on the overall data, patients after a Kasai porto-enterostomy seem to benefit from post-operative treatment with steroids. Clearance of jaundice at six months is significantly improved, and long-term transplantation-free survival increased. However, the optimal dosage of steroids still has to be determined.

This meta-analysis suggests that clinical outcome after Kasai might be improved using an intravenous, long term and high dosed regimen of steroids. However, this has to be confirmed by a large randomized study.

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CHAPTER 7

Macroscopically visible biliary discharge during Kasai: a prognostic factor?

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Submitted

Abstract

Kasai portoenterostomy is initially the best therapeutical option for biliary atresia. This study is to determine the prognostic significance of macroscopically visible biliary discharge during Kasai procedure. We analyzed thirty-six patients who underwent Kasai portoenterostomy for type III isolated biliary atresia in University Medical Center Groningen between 1986 and 2006 and had complete follow-up. The presence of biliary discharge was assessed retrospectively by reviewing operative records. Patients were divided into two groups: patients with per-operative biliary discharge (group I) and patients without per-operative biliary discharge (group II). There was no difference in age at Kasai, pre-operative liver function and liver fibrosis between both groups. Median serum total bilirubin levels at six months were 32 µmol/L (range 4 to 520) in group I and 55 µmol/L (range 8 to 483) in group II respectively (p=0.54). The percentage of patients with adequate clearance of jaundice, defined as total bilirubin below 20 µmol/l at six month after surgery, was similar in the 2 groups (~30%), as were the liver function tests at six months after surgery. After a median potential follow up of 5.6 years (range 1.3 to 20.8), overall survival was 75% in group I and 43% in group II (p = 0.04). Transplantation-free survival was 40% and 18% respectively (p=0.27). Mean overall survival was 15.6 ± 2.2 years in group I versus 10.6 ± 2.2 years in group II (p=0.11). Median transplantation-free survival was 1.6 years (95% CI, 0.01 to 1.2 years) and 0.6 years (95% CI 0.4 to 2.7 years), respectively. (p= 0.34). Our results show that macroscopically visible biliary discharge during Kasai is positively associated with survival.

Introduction

Biliary atresia (BA) is an obliterative disorder of unknown etiology that affects both the intra- and extrahepatic bile ducts. Currently, Kasai portoenterostomy is initially the best therapeutic option (1). Approximately, one third of patients after Kasai will survive with their native liver for over 10 years, another one third will need a liver transplantation before the age of 10 because of progressive liver cirrhosis, and the final third will need a liver transplantation at an early stage when adequate bile flow could not be restored (2). The large variability in long term success leads to consideration of predictors for success of the Kasai procedure. If the patients who are less likely to have a sustained successful response to Kasai procedure could be identified early, postoperative treatment modalities such as steroid therapy or prolonged postoperative use of antibiotics could be considered in these patients. So far, several predictors of Kasai procedure have been proposed, such as age at Kasai, the degree of fibrosis and the presence and diameter of bile ducts in the resected hilar plate (3-6). Surgical restoration of bile flow is widely thought to be the key to the success of Kasai. It is tempting to speculate that the presence of peroperatively visible bile flow predicts the success of the Kasai procedure. This study aims to test the prognostic significance of macroscopically visible biliary discharge as prognostic factor in patients undergoing Kasai portoenterostomy for biliary atresia.

Patients and methods

The records of thirty-nine patients who underwent portoenterostomy for isolated type III biliary atresia in University Medical Center of Groningen between 1986 and 2006 were available. Three patients were excluded from analysis since the presence or absence of biliary discharge could not be retrieved from the operative notes. All operations were performed by or supervised by four experienced pediatric and hepatobiliary surgeons. Liver fibrosis was scored by an experienced liver pathologist as mild (portal fibrosis without septa), moderate (portal fibrosis with septa) or severe fibrosis/cirrhosis (bridging fibrosis without cirrhosis/nodular transformation). After confirmation of the diagnosis by intraoperative

cholangiography, the fibrotic hilar plate was resected until grossly normal appearing liver tissue was reached. All patients were treated according to our postoperative protocol, which did not include the routine administration of steroids or antibiotics.

The presence of biliary discharge was assessed retrospectively by reviewing operative records. Biliary discharge was defined subjectively by the surgeon as either green fluid leaking from small bile ducts in the resectional plane or green discoloration of swabs. Patients were subsequently divided into two groups: group I with biliary discharge per-operatively and group II without biliary discharge per-operatively.

Primary end points were adequate clearance of jaundice, overall survival, and survival with native liver. Adequate clearance of jaundice was defined as serum total bilirubin below 20µmol/l at six month after surgery. The occurrence of cholangitis (fever associated with cholestasis) and portal hypertension and its sequelae (such as the presence of ascites, oesophageal varices, hypersplenism) were also assessed retrospectively from the charts.

Data were analyzed by using Mann Whitney U test for continuous data and Fisher's exact test for categorical data. Survival curves were constructed using the Kaplan Meier method, and compared with the log rank test. A P value <0.05 was considered significant, and 95% confidence intervals were quoted where appropriate. Due to the low number of patients multivariate analysis was not performed.

Results

Of the thirty-six patients who underwent portoenterostomy for type III biliary atresia, there were 17 males and 19 females. Group I (with discharge) was composed of 20 patients, 8 male and 12 female, and group II (without discharge) of 16 patients, 9 male and 7 female.

Mean age at Kasai was 66±21 days in group I and 73±28 days in group II ($p=0.38$). There was no difference in the grade of fibrosis at the time of surgery (severe fibrosis in 46% and 50% resp.)

Median serum total bilirubin levels at six months were 32 µmmol/L (range 4 to 520) and 55 µmol/L (range 8 to 483), respectively ($p=0.54$). The percentage of patients

with adequate clearance of jaundice was 29% (with discharge) and 31% (without discharge), respectively. The two groups did neither differ in pre-operative, nor in postoperative liver functions tests at six months after surgery (Table 1).

The median potential follow up was 5.6 years (range 1.3 to 20.8), and there was no difference in the duration of follow up between both groups. Overall survival was 75% in group I and 43% in group II (p = 0.04). Transplantation-free survival was 40% and 18% respectively (p=0.27). Mean overall survival was 15.6 ± 2.2 years in group I versus 10.6 ± 2.2 years in group II (p=0.11, see figure 1). Median transplantation-free survival was 1.6 years (95% CI 0.01 to 1.2 years) and 0.6 years (95% CI 0.4 to 2.7 years) respectively (p=0.34, see figure 2). Survival after transplantation was 66% in group I and 25% in group II. (p=0.08). As the UMCG is the only pediatric transplant center in the Netherlands, access to orthotopic liver transplantation (OLT) was uniform throughout the study period. There was no difference in survival with native liver between surgeons, nor was there a difference between patients operated before or after 2000. There was no difference in the incidence or the time of first occurrence of cholangitis or portal hypertension between both groups.

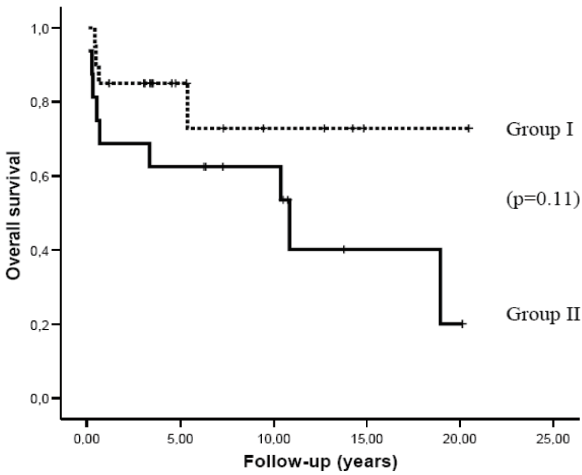

Figure 1. Overall survival after Kasai porto-enterostomy in patients with (Group I) and without biliary discharge (Group II) during the operation.

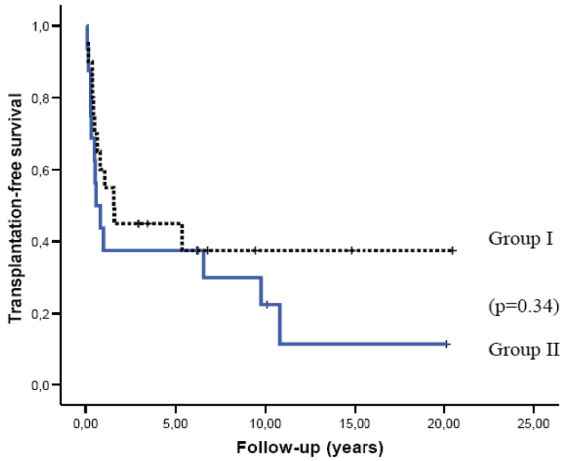

Figure 2. Transplantation-free survival in patients with (Group I) and without biliary discharge (Group II) during the operation.

Discussion

In the present study, overall survival is significantly higher in patients with visible biliary discharge during surgery compared with patients without. A similar trend is found in transplantation-free survival and overall survival time, even though no statistical significance is shown. The presence of visible biliary discharge during Kasai might therefore be a prognostic factor.

One of the short term predictors of sustained effectiveness of Kasai procedure is resolution of jaundice. If jaundice persists after operation, patients are likely to develop liver failure. In the present study there was no significant difference in the clearance of jaundice at six month. When this is compared with the transplantation-free survival curves, this suggests that the difference between patients with and without biliary discharge becomes apparent in a later stage, sometimes even after liver transplantation. This is also suggested by overall survival after liver transplantation in this patient group (66% in patients with and 25% in patients without biliary discharge). The mechanisms behind this phenomenon are as yet unknown, but limited numbers prevent us from exploring this any further.

Davenport et al (7) introduced a semiquantative scoring system, the “macroscopic appearance at portenterostomy (MAP) score” This includes macroscopic aspects such liver consistency, size of portal remnants, presence of portal hypertension and extrahepatic anomalies. A correlation was determined between MAP score and the

sustained results of Kasai. In this study, the macroscopic aspect of the biliary remnants was the most important prognostic factor, even more so than age. This is in accordance with the results of the present study. Others have demonstrated the microscopic appearance of the biliary remnants to be of prognostic importance: few portal biliary remnants and absence of portal inflammation were identified as valuable predictors for poor outcomes (8-12). In view of the above, careful peroperative observation by an experienced surgeon could be valuable in predicting the success of portoenterostomy. The presence of extrahepatic anomalies may not be a prognostic factor (13).

Other prognostic factors that have been suggested include age at operation, liver fibrosis and type of BA. Clearance of jaundice at six weeks or three months may also be predictors for long term outcome (14, 15). A Japanese study (16) introduced a multivariate linear model for calculation of BA prognostic index, which included nine serum parameters, such as albumin concentration (ALB), total bilirubin (TB). However, this is not easily used in daily practice.

The key of successful Kasai is to restore bile drainage, thus inhibiting the progression of biliary injury leading to cirrhosis and liver failure (17). Meticulous surgical technique is paramount in achieving adequate bile drainage, starting with a wide exposure of the porta hepatis (18-20). Theoretically, a more extended dissection into normal tissue levels until biliary discharge is reached may improve outcomes. However, supra-portal dissection, which is the resection of the portal fibrous mass until normal bile ducts are reached, results in a significantly decreased success rate of biliary drainage and diminished survival (21-23). This supports the concept that the small remaining bile ducts in the portal fibrous mass with continuity to the intrahepatic biliary tract are the main sources of biliary drainage after Kasai. The results of the present study also support this theory, as visible biliary discharge might be considered proof of patent bile ducts in the resectional plane.

Patients without visible discharge were slightly older (73 days) than patients with visible discharge (66 days) at the time of Kasai, but this did not reach statistical significance. The two groups also did not differ in the length of the Roux-Y limb. We did not assess other possible prognostic factors such as size of biliary remnants or degree of inflammation at the porta hepatis. Given the low number of patients, multivariate analysis would be impossible.

This is a retrospective study with all its limitations. However, given the consistency of the results over the years, without differences between experienced pediatric surgeons, we consider the presence of biliary discharge as a prognostic factor for patients undergoing Kasai porto-enterostomy for biliary atresia. Of course, this is a prognostic factor that becomes apparent only during laparotomy. Other - pre-operative - prognostic factors identifying patients who will benefit from Kasai should be identified to optimise treatment for these patients.

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CHAPTER 8

Summary, general discussion and future perspectives

Summary

Chapter 1 provides an introduction of human biliary atresia (BA). Furthermore, the aims of this thesis are introduced: to investigate hepatobiliary transport and liver fibrogenesis in the development of biliary atresia in a mouse model; and to evaluate several clinical aspects of human BA and other pediatric cholestatic liver diseases.

In **part I** of this thesis, we focused on several aspects in the development of murine BA using the rhesus rota virus (RRV)-induced murine BA model. In **Chapter 2**, the literature regarding the animal model of BA and RRV-induced murine BA was reviewed. RRV-induced murine BA is a valid animal model for investigating human BA, as it reproduces the key clinical and histomorphologic features of human BA. It is due to a viral infection of the biliary epithelium followed by secondary immune and autoimmune mediated biliary obliteration. There is a prominent Th1 response in the early phases of BA. Inactivation of IFN-g can prevent biliary obstruction not initiation of inflammation. Apoptosis activated by TNF-a and IFN-g seems to be one of the most important mechanisms resulting in bile duct injury.

The fibro-proliferative to liver injury is well characterized in humans. However, little is known about this response in the murine BA model, as most studies focus on immunological aspects of the model and not on the response to injury. As the Hedgehog (Hh) signalling and epithelial mesenchymal transition (EMT) are involved in the liver fibro-proliferative response in several other cholestatic liver diseases, in **Chapter 3** we aim to test the hypothesis that liver fibrosis occurs early during biliary atresia development, and to demonstrate the presence of epithelial to mesenchymal transition and Hedgehog signalling during this process.

The same RRV-induced murine BA model was used. Seven days after viral inoculation, while there was only inflammation but no bile duct obstruction, fibrosis could already be demonstrated by periportal accumulation of aSMA positive myofibroblast cells with increasing mRNA levels of aSMA, type I collagen and Tgf-β_1. At the same time, ductular cells started to express the epithelial-mesenchymal transition marker *S100a4*. Periportal accumulation of ductular cells expressing Indian Hedgehog (but not Sonic Hedgehog), the receptor Patched and the Hh target gene *Gli2* was found. After 14 days biliary stenoses were visible, which concurred with an increase in serum conjugated bilirubin, indicative for extrahepatic

bile duct obstruction. The fibro-proliferative process progressed, accompanied by increased levels of the epithelial to mesenchymal and Hedgehog parameters. The conclusion is that during the development of biliary atresia, the fibro-proliferative response occurs early, even before bile duct obstruction. During this fibro-proliferative response, epithelial to mesenchymal transition and hedgehog signalling activity can also be observed at the early stages. Further investigations should be aimed at elucidating the causal relations between Hedgehog signalling, epithelial to mesenchymal transition and the fibro-proliferative response in this model.

We hypothesized that the early development of intrahepatic cholestasis could be another reason of the early occurrence of fibro-proliferative response and liver fibrosis in this model. Therefore, in **Chapter 4**, we aimed to demonstrate that in the early development of BA changes in hepatobiliary transport are mainly related to the inflammatory process and not to extrahepatic cholestasis. These changes in hepatobiliary transport lead to intrahepatic cholestasis and subsequent liver injury. In the RRV induced murine model of BA, liver and blood samples were collected and studied at seven, 14 and 18 days. Seven days after inoculation, visible jaundice occurred. Despite a high bile acid concentration in the liver, gene expression of canalicular and basolateral hepatobiliary transporters and their regulatory nuclear receptors was down-regulated. This was paralleled by an increase in gene expression of inflammatory cytokines. At 14 days, hepatobiliary transporters and nuclear receptors remained downregulated although the inflammatory response subsided and rhesus rotavirus proteins decreased. The percentage of conjugated bilirubin started to increase as extrahepatic biliary obstruction occurred. At 18 days, gene expression of hepatobiliairy transporters remained low, expression of nuclear receptors returned to normal, while expression of inflammatory cytokines decreased further. Histology demonstrated progressive inflammation, bile duct damage and proliferation and hepatocyte necrosis. The conclusion of this study was that intrahepatic cholestasis due to down-regulation of basolateral and canalicular hepatobiliary transporters is an early event in the development of biliary atresia. This down-regulation is probably due to the inflammatory process, which apparently overrides the regulatory effects of the concomitant increase in bile acids in the liver. The net result is intrahepatic cholestasis and liver damage, which occurs already before extrahepatic biliary obstruction. The results of the present study are in line with preliminary studies in human, offering further proof of the validity of the mouse

model and opening research approaches to mechanistical studies and possible therapeutic interventions.

In **part II** of this thesis, we focussed on several clinical aspects of human BA and other pediatric cholestatic liver diseases. In **Chapter 5**, we aimed to determine the long term outcomes of partial external biliary diversion (PEBD) in the treatment of progressive familial intrahepatic cholestasis (PFIC) and Alagille's disease. A retrospective chart review was performed of all 14 (PFIC 11, Alagille's disease 3) patients undergoing PEBD in the University Medical Center of Groningen (UMCG) from 2002 to 2005. Median follow-up was 3.1 years (range 2.0 to 5.7). One patient (7%) underwent a liver transplantation at 3.2 years post PEBD. Two years postoperatively 50% were without pruritus, 21% had mild pruritus. In 29%, pruritus had not diminished, and three of these patients had severe fibrosis pre-operatively. In patients with mild/moderate fibrosis, PEBD decreased serum bile salts (105µmol/L (range 8-269) two years postoperatively). Bile salts did not decrease in patients with severe fibrosis. The conclusion of this study was at median follow-up of 3.1 years after PEBD, pruritus has been relieved in 75%. Bile salts level and growth were improved in most of the patients. Longer follow-up is needed to determine whether PEBD can avoid or merely postpone the demand for liver transplantation.

In **Chapter 6,** we aimed to determine the possible role of treatment with steroids after Kasai porto-enterostomy. A systematic review was performed of the English literature published between 1995 and January 2008 with emphasis on the differences in outcome between patients with or without post-Kasai steroid treatment. Main outcome parameters were clearance of jaundice at six months; 1-year, 3-year and 5-year survival, and incidence of cholangitis. Twenty studies were included: one randomized trial, one prospective single-centre study, seven retrospective comparative studies, and eleven retrospective non-comparative studies. Overall, clinical outcome parameters were analyzed from 1175 patients with and 645 patients without steroids treatment. The percentage of patients with adequate clearance of jaundice was significantly higher in the steroids group (60% vs. 40%; odds ratio 2.16, 95% CI 1.08 – 4.35). Transplantation free survival seemed also to be increased in the group of patients receiving steroids (1yr, 65% vs. 51%; 3yr, 72% vs. 30%; and 5 yr, 58% vs. 36%, respectively). The three-year

survival difference was statistically significant (p=0.03, odds ratio=6.0, 95% CI 1.40-26.50). There is a discrepancy between the results of the only (small) randomized trial which does not show a benefit of steroids and the combined results of the other studies which do demonstrate such a benefit of steroids treatment after Kasai. Based on the overall data, patients after a Kasai portoenterostomy seem to benefit from post-operative treatment with steroids especially by an intravenous, long term and high dosed regimen of steroids. However, this has to be confirmed by a large randomized study.

In **Chapter 7**, we aimed to identify the presence of macroscopically visible biliary discharge during Kasai procedure as a prognostic factor for the outcome of the procedure. Thirty-six patients who underwent Kasai portoenterostomy for type Ш BA in UMCG between 1986 and 2006 with complete follow-up were analyzed in this study. Patients were divided into two groups: patients with per-operative biliary discharge (group I) and patients without per-operative biliary discharge (group II).There was no difference in age at Kasai, pre-operative liver function and liver fibrosis between both groups. Median serum total bilirubin levels at six months were 32 µmol/L (range 4 to 520) in group I and 55 µmol/L (range 8 to 483) in group II resp (p=0.54). The percentage of patients with adequate clearance of jaundice, defined as total bilirubin below 20 µmol/l at six month after surgery, was similar in the 2 groups (~30%), as were the liver function tests at six months after surgery. After a median potential follow up of 5.6 years (range 1.3 to 20.8), overall survival was 75% in group I and 43% in group II (p = 0.04). Transplantation-free survival was 40% and 18% resp (p=0.27). Mean overall survival was 15.6 ± 2.2 years in group I versus 10.6 ± 2.2 years in group II (p=0.11). Median transplantation-free survival was 1.6 years (95% CI, 0.01 to 1.2 years) and 0.6 years (95% CI 0.4 to 2.7 years), resp. (p= 0.34). Therefore, we concluded that macroscopically visible biliary discharge during Kasai is positively associated with the overall survival.

General discussion and future perspectives

Part I: Different aspects in the development of biliary atresia in an animal model

In this section, we firstly summarized the current concepts of the most widely used animal model of BA, the rotavirus-induced BA mouse model. RRV infects the cholangiocytes thus inducing a pan-cholangitis, followed by an (auto) immune reaction aimed at the biliary epithelium.

Although many studies focus on the immunological mechanisms, there is little data on the response of the liver to the injury to the biliary epithelium. We therefore first investigated hepatobiliary transport regulation and its relation to the inflammatory response, the development of intrahepatic cholestasis and liver injury. We demonstrated a temporal relation between inflammation and bile transport, with bile transport decreasing even before bile duct obstruction occurs. Although we have demonstrated a temporal relation between inflammation and bile transport, we can only speculate on the mechanisms involved in this relation. Further studies should focus on the mechanisms via which inflammation and hepatobiliary transport are related and on the functional effects of these changes in bile composition.

In addition, nuclear receptors are considered promising therapeutical targets. Studies in rodents revealed that *Fxr* is an important factor determining liver injury during cholestasis (1-4). The use of synthetic *Fxr* agonists in cholestatic rodent models has resulted in a decrease in biochemical and histological markers of liver injury. Hence, the effects of FXR agonists in the earlier stages of BA development – in which there is no complete bile duct obstruction - would be worth investigating.

We have studied the effects on biliary transport in the hepatocyte. But as the cholangiocyte is considered to be the primary target of RRV in the murine BA model (5), observing the impact of RRV on cholangiocyte transporters would help us to improve our understanding of this model. This might be studied in vitro, using cholangiocyte cell lines or freshly isolated cholangiocytes from the RRV-induced murine BA model.

Next, we investigated Hh signaling activity, EMT and the liver fibro-proliferative process in the murine BA model. We found that the fibro-proliferative response starts early, with signs of fibrosis present already one week after inoculation, which is even before bile duct obstruction occurs. Hh signaling and EMT parallel the liver fibro-proliferative process. However, we do not know what happens before seven days, the sequence of the above events. Therefore, we need to set up earlier and more frequent time points in next experiment, which would give us more information about this process of liver repair. Moreover, human samples of various stages of BA need to be studied as well to get insight about the role of Hh and EMT at advanced stages in which biliary obstruction has already completely developed and inflammatory response has already subsided.

The heterozygously deficient rats for the Hh receptor *Ptc* (PtcLacZ rats) following bile duct ligation (BDL) display an altered liver repair response to BDL with expanded and increased fibroductular response and poorly organized ductular structure (6). Hence, it would be interesting to investigate the impact of blocking the Hh pathway to the liver response in the development of BA. This might offer insight into the mechanisms by which Hh might influence the fibro-proliferative response.

One of those mechanisms is the induction of epithelial-mesenchymal transition (EMT) by Hh signalling to repair the liver. In our study, we found that EMT occurred on ductular cells when Hh signalling is activated. Another promising investigation is to study further on the cholangiocytes isolated from the BA mice livers at different time points, so that we could evaluate precisely the process of EMT and the role of Hh in the regulation of EMT.

Due to the limited survival of the animals, a more prolonged study was impossible. This makes comparison with human data difficult, as human specimens display a more advanced stage of the disease. Therefore, it would be very interesting to prolong the survival of BA mice. In our study, most pups suffering BA died of dehydration due to insufficient feeding from their mother. One possible way to avoid feeding problems and thus increase the survival of the animals is artificial feeding. One way of artificial feeding is using the so-called “pup in the cup model”. In this model, the newborn pups are artificially reared through a surgically placed gastrostomy catheter (7). Via this catheter different feeding regimens may be tested, offering the possibility to investigate the effects of dietary measures on the progress of BA.

Part II: Clinical aspects of human biliary atresia and other pediatric cholestatic liver diseases

In this section, we firstly aimed to investigate the long term outcomes of PEBD in the treatment of two other pediatric cholestatic disorders, PFIC and Alagille's disease. We also investigated quality of life after this procedure. We concluded that at after a median follow-up of 3.1 years, pruritus has been relieved in 75%. Bile salts level and growth are improved in most of patients. However, follow-up is still relatively short and patient numbers are low. Moreover, the patients with severe fibrosis pre-operatively have less favourable postoperative outcomes without improvement on symptoms and bile salts levels, compared with the patients with mild or moderate fibrosis. With the increasing success rates of liver transplantation in children, some centres advocate liver transplantation as a first option for children with PFIC(8). Although results of liver transplantation have improved enormously, we only favor this approach in patients with severe fibrosis or cirrhosis as PEBD does not preclude liver transplantation in a later stage. The good results of PEBD in the absence of severe fibrosis/cirrhosis in combination with the scarcety of donor organs, the costs of livertransplantation and the necessity for life-long immunosuppressive medication make PEBD the first surgical option. However, follow-up should be continued to assess long term follow-up. From a scientific point of view routine liver biopsies to assess the progression of liver fibrosis could be considered, although there is little gain for clinical decisionmaking.

Next, we performed a meta-analysis to determine the possible beneficial impact of steroids treatment after Kasai. In this study, we got a discrepancy between the overall results which suggests a benefit for using steroids and the only randomized trial (on a limited number of patients and with a relatively low dose of steroids) which does not. A large-scale, multi-centre, high dose, placebo-controlled and double-blinded randomized trial is therefore highly in demand. At present, the Biliary Atresia Research Consortium (BARC) is conducting a clinical trial to evaluate whether long-term, high dose treatment with steroids improves the outcomes of Kasai in patients with BA among ten major medical centres in US.

Lastly, we found macroscopically visible biliary discharge during the Kasai procedure to be positively associated with the overall survival and transplantation-free survival. However, this prognostic factor needs to be validated before it comes to clinical application. The criteria of macroscopically visible biliary discharge should be clearly and strictly defined. For instance, how to objectively identify the bile from other body fluids? How to define the "macroscopically visible"? Is the definition of volume of bile flow or the contents and consistency of bile flow more important?

In summary, new insights are provided into the development of murine BA and clinical aspects of human BA and other pediatric cholestatic diseases. We demonstrated that the hepatobliary transport downregulation is mainly attributed from the inflammation instead of extrahepatic cholestasis at the early stage of the development to BA. This leads to intrahepatic cholestasis. We showed that Hh signalling activation and EMT are involved in the early fibro-proliferative response during the development of murine BA. These observations are new insights into the mechanism of progressive liver fibrosis in BA. Moreover, we summarized the experience of UMCG on PEBD to conclude that PEBD is an effective procedure in the treatment of two other pediatric cholestatic diseases, PFIC and Alagille's disease. In addition, our systematic review suggested that patients who underwent a Kasai portoenterostomy seem to benefit from post-operative treatment with steroids. This meta-analysis is so far the best evidence available. Finally, the investigation on macroscopically visible biliary discharge during Kasai as a prognostic factor opened a new aspect for clinicians to predict the outcomes of Kasai procedure.

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Nederlandse Samenvatting

Samenvatting

In dit proefschrift worden verschillende aspecten van galwegatresie en andere cholestatische ziekten op de kinderleeftijd onderzocht.

Deel I richt zich op de pathogenese van galwegatresie. Hierbij wordt gebruik gemaakt van een diermodel waarbij bij pasgeboren muizen ten gevolge van infectie met rotavirus galwegatresie ontstaat. Allereerst wordt een overzicht gegeven van dit model. Vervolgens wordt, gebruik makend van dit diermodel, aangetoond dat leverfibrose al in een vroeg stadium optreedt. De fibrose gaat gepaard met epitheliale naar mesenchymale transitie en activatie van Hedgehog signaaltransductie. De fibrose treedt al op voor de galwegobstructie.

Daarna zijn de effecten van ontsteking op hepatobiliair transport onderzocht. Het diermodel maakt gebruik van infectie van de cholangiocyt met rotavirus. Voor galweg obstructie ontstaat, ontstaat een inflammatoire reactie. Deze ontstekingsreactie leidt tot down-regulatie van hepatobiliare transporteiwitten, wat vroegtijdige intrahepatische cholestase tot gevolg heeft. Dit is mogelijk een van de redenen voor de vroeg optredende fibrose.

In deel II worden klinische aspecten van galwegatresie en andere cholestatische ziektebeelden onderzocht. In het eerste hoofdstuk worden de lange termijn resultaten van partiële externe biliaire diversie bij kinderen met progressieve familiaire intrahepatische cholestase en het syndroom van Alagille onderzocht. Deze behandeling blijkt bij kinderen zonder ernstige fibrose in het pre-operatieve biopt succesvol.

Uit een meta-analyse blijkt vervolgens dat kinderen na een operatie voor galwegatresie mogelijk baat hebben bij hoge doseringen steroïden. Uit een retrospectieve studie naar kinderen met galwegatresie blijkt tenslotte dat macroscopisch zichtbare galafvloed tijdens de operatie een gunstige prognostische factor is bij kinderen die een Kasai-portoenterostomie ondergaan.

中文摘要

中文摘要

在本论文中，我们研究了与胆道闭锁相关的多个方面和其它儿童黄疸性疾病。

我们主要研究胆道闭锁的病理机制。胆道闭锁的动物模型是通过采用轮状病毒注射新生小鼠建立的。首先，对这一动物模型相关的研究进行了系统回顾。然后，在这个动物模型基础上，证实了肝纤维化开始于胆道闭锁发展的早期。肝脏纤维化与上皮细胞向肝实质细胞的转化和 Hedgehog 信号通路的激活同时发生。在胆道梗阻形成之前，肝脏纤维化的过程已经发生。随后，对炎症对肝胆运输基的影响作了研究。炎症导致肝胆运输基相关基因下调，从而导致了肝内黄疸。这是导致肝脏纤维化早期发生的主要原因。

在论文的第二部分，对胆道闭锁和其它儿童黄疸性疾病的临床方面做了研究。在这部分的第一章中，回顾了部分胆道外引流治疗进展性家族性肝内黄疸性疾病和 Alagille 综合征的长期结果。研究结果证明这种手术对术前肝脏活检没有纤维化的儿童取得很好的效果。

Meta 统计分析证明胆道闭锁经手术治疗后的患者大剂量服用类固醇类药物可能会取得较好的术后效果。回顾性研究胆道闭锁经手术治疗的临床资料，证实术中可见胆道引流是影响术后预后的因素。

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Curriculum Vitae

Curriculum Vitae

Huiqi Yang was born on June 23rd, 1975 in Tainjin, China. After six years' primary school studying at Nanmenxi Primary School, she went to the 25th Junior high school in 1991. After three years' study, she had the opportunity to study in one of the best high schools in Tianjin (Yaohua high school). In 1999, she graduated from Yaohua high school and was enrolled by Tianjin Medical University. After five years' industrious studying, she began her clinical rotation for her residency at Nankai Hospital of Tianjin Medical University in 1999. In 2001, she got the opportunity for her surgical training at the same hospital. At the same time, she passed the Master exam in Tianjin Medical University and did her Master study under the supervision of Professor dr. Mingfang Qin. Her Master thesis was about the surgical treatment of gastro-esophageal reflux disease, and she completed her Master study in 2003. In 2004, she passed her PhD exam in Tianjin Medical University, and continued her clinical research about gastro-esophageal reflux disease for her PhD research under the supervision of Professor dr. Xianzhong Wu. In 2005, she fulfilled her surgical training and specialized in the field of Upper GI and Abdominal Surgery. During the period of 2006 to 2007, she was a visiting surgeon in Upper GI Surgery in Flinders Medical Centre in South Australia under the supervision of Professor dr. D.I. Watson, and worked out two English publications about surgical treatment of gastro-esophageal reflux disease. Due to the cooperation between Tianjin Medical University and Groningen University, she studied as a PhD student from 2007 to 2009 in Surgery Department of University Medical Centre Groningen in the Netherlands. The upcoming year, Huiqi will continue her clinical work as a qualified surgeon and do part-time research in Upper GI field.

List of publications

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